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 EFENDIĆ S & JOHANSSON C Hormonal  
 release to the gastric lumen in man



## ULF von EULER 75 YEARS

On his 75th birthday on February 7th Ulf von Euler may look back on an exceptionally successful scientific career. During half a century he and his collaborators have delivered a steady stream of scientific publications which early secured him a leading position in international physiology. Certainly he had some advantages. He grew up in a scientific milieu—his father Hans von Euler became Nobel laureate in chemistry—he had excellent teachers in science—Cornelius Heymans in Gent, Henry Dale in London, Goran Liljestrand in Stockholm—and a stimulating working place in Stockholm with Goran Liljestrand and Yngve Zotterman as highly qualified collaborators. But the key to Ulf von Euler's success in science lay in his personal qualities. With the intuitive talents of the born scientist he selected essential problems and tackled them with consistency and intensity. In this way he has made a series of now famous discoveries. Especially worth mentioning are his early observations—already in the 30-ties—on substance P and prostaglandin, contributions which showed to be of paramount importance for later developments. The top of his scientific production was reached by the discovery of noradrenaline as the chemical mediator at postganglionic sympathetic nerve terminals, an achievement which rendered him the Nobel Prize in physiology or medicine in 1970.

Ulf von Euler is since 1957 chief editor of *Acta Physiologica Scandinavica*. Under his eminent leadership the journal has flourished and its international reputation has become well established.

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# Excess cumulative blood flow and repayment during reactive hyperemia in human cutaneous tissue

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KRISTENSEN J. K. & HENRIKSEN O. Excess cumulative blood flow and repayment during reactive hyperemia in human cutaneous tissue. *Acta Physiol Scand* 1980; 108: 1-6. Received 30 Oct. 1978. ISSN 0001-6772. Department of Dermatology, University of Copenhagen, Rigshospitalet, and Medical Department II, City Hospital, Copenhagen, Denmark.

The influence of duration of vascular occlusion upon the reactive hyperemic response in human cutaneous tissue was studied in 6 subjects. Blood flow in cutaneous tissue was measured dorsally on the distal phalanx of the second finger by the local  $^{133}\text{Xe}$  washout technique. Post-occlusive blood flow, calculated from the steepest part of the  $^{133}\text{Xe}$  washout curve just after release of vascular occlusion, reached a maximum value when duration of vascular occlusion was 1 min. However, excess cumulative blood flow, i.e. the integrated blood flow during reactive hyperemia minus integrated pre-ischemic blood flow for a period corresponding to the duration of the reactive hyperemic response, increased with increasing duration of vascular occlusion from 3 to 24 min. Fractional repayment, excess cumulative blood flow divided by pre-ischemic blood flow times duration of vascular occlusion, was not correlated significantly to duration of vascular occlusion. However, there was significant inverse correlation between fractional repayment and pre-occlusive blood flow, indicating that, besides metabolic factors, pre-ischemic blood flow in cutaneous tissue is influenced by other factors, such as local regulation.

**Key words:** Reactive hyperemia, cutaneous tissue, normal fingers.

The phenomenon of reactive hyperemia in response to vascular occlusion has been studied in many different tissues and organs: arm and leg segments (Shepherd 1930, 1964; Patterson 1956); hand (Krog *et al.* 1960); finger (Patel & Burton 1956); skeletal muscle (Montgomery *et al.* 1934; Hahm 1953; Lundberg 1966); myocardium (Collman & Gregg 1960, 1961); liver (Hannon & Johnson 1966); kidney (Hooda *et al.* 1968); and adipose tissue (Nielsen & Seyn 1977; Henriksen *et al.* 1976).

Common findings are that blood flow increases following vascular occlusion and that excess occlusive blood flow is the integrated blood flow during the period of reactive hyperemia minus the integrated pre-ischemic blood flow for a period of the same duration increases with duration of vascular occlusion.

No information on the hyperemic response to vascular occlusion in cutaneous tissue has so far been provided.

In fingers, the absolute amount and duration of

extra blood flow increased with the period of occlusion. The blood flow debt repaid showed a large variation but amounted on average only to 30% (Patel & Burton 1956). However, since blood flow was measured by venous occlusion plethysmography, the contribution of post-ischemic hyperemia in tissues other than skin is included and the results may be influenced by changes in blood flow through arterio-venous anastomoses. Using the local  $^{133}\text{Xe}$  washout technique, it is possible to measure microvascular blood flow in cutaneous tissue. The purpose of the present study was to investigate the influence of duration of vascular occlusion and of pre-ischemic blood flow level upon excess cumulative blood flow and repayment in cutaneous tissue.

## EXPERIMENTAL PROCEDURE

The experiments were carried out on 6 healthy persons aged 34-46 years. Room temperature was kept constant at 24°C. During the experiments, the subjects were seated on

The number of subscribers have more than trebled—they are today ~1700 and—quite outstanding for a scientific journal—the economy is good in fact good enough to allow an annual contribution to be distributed as travel stipends among young Scandinavian scientists attending Scandinavian physiology meetings. For the success of *Acta Physiologica Scandinavica* we have to thank Ulf von Euler—and his wife and collaborator Dagmar—for their unselfish and untiring editorial work and care for *Acta*. We collaborators and contributors, subscribers and readers all congratulate our chief editor on his 75th birthday and wish him still many active years.

*Birge Ulfar*

## Excess cumulative blood flow and repayment during reactive hyperemia in human cutaneous tissue

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Common findings are that blood flow increases following arterial occlusion and that excess cumulative blood flow, i.e. the integrated blood flow during the period of reactive hyperemia minus the integrated pre-ischemic blood flow for a period of the same duration, increases with duration of arterial occlusion.

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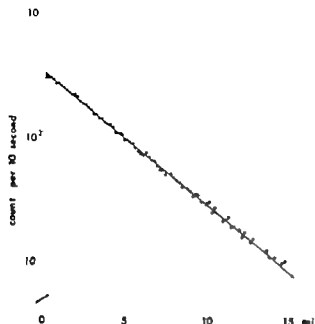


Fig. 1. Undisturbed Xenon washout curve from the dorsum of the distal phalanx.

an adjustable chair, the legs pendant, the arm and hand resting at heart level. Cutaneous temperatures on the dorsum of the finger were recorded by thermocouple during the experiments.

A skin area of 1 cm diameter just proximal to the nail fold on the dorsum of the second or third left finger was labelled with  $^{133}\text{Xe}$  using the atraumatic epicutaneous labelling technique (Sejrsen 1971). A NaI scintillation detector collimated to record only from the labelled area and its immediate surroundings was placed 5 cm above the labelled area. The detector was connected to a printing gamma spectrometer with a window set around the 0.081 MeV photopeak of Xenon. The activity was printed out at 10 s intervals. A miniature blood pressure cuff 70 mm wide connected to a mercury manometer was applied around the proximal phalanx of the finger. Immediately after a labelling period of 5 min the washout of tracer was followed for 4 to 5 min. Then suddenly the blood pressure cuff was inflated to 300 mmHg inducing ischemia of the finger. A vascular occlusion was maintained for periods of 3.6 l or 4 min. After release of the pressure in the cuff the washout of Xenon was followed until background activity was reached.

The count rate versus time were plotted on semi-logarithmic paper after subtraction of background activity. In order to study the undisturbed washout of  $^{133}\text{Xe}$  from the region under study, separate washout curves were obtained in all six subjects with inducing vascular occlusion (Fig. 1).

## CALCULATIONS

The following calculations are based on the assumptions of homogeneous perfusion, no recirculation of tracer and flow-limited washout of tracer.

A typical reactive hyperemia Xenon washout curve shown in Fig. 2.

1. *Control blood flow* ( $f_{\text{con}}$ ) was calculated from the initial monoexponential portion of the washout curve according to the formula  $f_{\text{con}} = k_{\text{con}} \lambda 100$  (ml/min/100 g) (Kety 1951), where  $k_{\text{con}}$  denotes the washout rate constant (min<sup>-1</sup>) and  $\lambda$  the blood to tissue partition coefficient (ml/g). A  $\lambda$  value of 0.7 ml/g was used for cutaneous tissue (Sejrsen 1971).

2. *Maximum blood flow* ( $f_{\text{max}}$ ) was based on the washout rate constant  $k_{\text{max}}$  calculated from the steepest initial part of the  $^{133}\text{Xe}$  washout curve following deflation of the cuff  $f_{\text{max}} = k_{\text{max}} \lambda 100$  (ml/min/100 g).

3. *Cumulative blood flow* ( $V_{\text{cum}}$ ) is the integrated blood flow in the hyperemic period) was calculated for the period  $t \rightarrow t'$  (cf. Fig. 2) as follows:

$$V_{\text{cum}} = \int_t^{t'} f(t) dt \quad (1)$$

where  $f(t)$  is the declining perfusion coefficient in ml/min/100 g. Under the above mentioned assumption the externally monitored fractional decrease in activity at time  $t$   $-dq(t)/dt/q(t)$  is given as

$$(-dq(t)/dt)/q(t) = f(t)/\lambda \quad (2)$$

where  $q(t)$  is the activity at time  $t$ . Rearranging equation (2) and integrating on both sides give

$$\lambda \int_{q(t)}^{q(t')} \frac{d \ln q(t)}{q(t)} = \int_t^{t'} f(t) dt = V_{\text{cum}} \quad (3)$$

with the solution

$$V_{\text{cum}} = \lambda \ln [q(t')/q(t)_{\text{con}}] \quad (4)$$

4. *Initial maximal blood flow*  $V_0$  was determined as cumulative blood flow during the period of reactive hyperemia ( $t \rightarrow t'$ ) minus cumulative post-hyperemic blood flow for a period of same duration. This gives

$$V_0 = \lambda \ln [q(t')/q(t)_{\text{con}}] \quad (5)$$

where  $q(t)_{\text{con}}$  is obtained by extrapolating the activity versus time from  $t'$  to  $t$  (cf. Fig. 2).

4. *True initial exposure*  $R$  equal  $V_0$  divided by cumulative blood flow deficit  $V_{\text{def}}$  (the product of  $f$  and the duration of vascular occlusion) is

$$R = V_0/V_{\text{def}} (\lambda \text{ l } t^{-1}) \quad (6)$$

Student's  $t$  test for paired or unpaired samples were used. A limit of significance was chosen 0.05.

## RESULTS

The background-corrected washout curve obtained in control experiment had a monoexponential course over about 15 sec (Fig. 1).

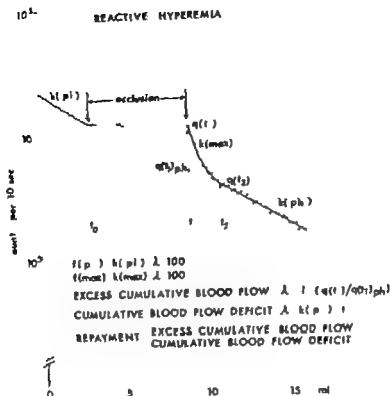


Fig 2 An example of <sup>125</sup>Xe washout curve.  $ph$  pre-ischemic,  $ph$  posthyperemic

An example of the <sup>125</sup>Xe washout curve is shown in Fig. 1. The experiments a washout rate constant,  $k_{max}$ , was obtained in a post-hyperemic period which did not differ significantly from that ( $k$ ) of the pre-ischemic period ( $P < 0.3$ ). Mean pre-ischemic blood flow  $1 \pm 1$  S.E. was  $8.9 \pm 4.4$  ml/min/100 g and post-hyperemic blood flow  $2.6 \pm 0.8$  ml/min/100 g (mean of 19 experiments).

Mean blood flow increased when the duration of ischemia was prolonged from 3 to 12 min. Prolongation of the duration of vascular occlusion to 4 min did not cause further increase in maximum blood flow (Fig. 3).

Duration of reflow. A hyperemia increased from 0.8 (0.1 min) to 4.0 (9 min), when the period of vascular occlusion was prolonged from 3 to 4 min (Fig. 4).

End-arterial blood flow increased when the duration of vascular occlusion was prolonged from 3 min to 4 min but with smaller increment for the period 1 to 4 min than for the period 2 to 12 min (Fig. 5).

Fractional repayment. This was on an average 82, 73, 72 and 69% after 3, 6, 1 and 4 min of occlusion. The values plotted versus pre-ischemic blood flow are shown in Fig. 6.

## DISCUSSION

The described method for calculation of cumulative blood flow is based on the assumption that homogeneous perfusion flow limited washout of tracer and absence of tracer recirculation apply to the area studied by <sup>125</sup>Xe washout. This assumption seems valid since the washout of <sup>125</sup>Xe in cutaneous tissue shown in the control experiment (Fig. 1) followed a monoexponential course to a level where remaining activity only amounted to about 1% of the initial amount of activity. This finding can be explained by the observed absence of subcutaneous tissue in the region studied (Hale & Burch 1960).

When furthermore the rest of the hand is shielded off detection of tracer accumulated in fat

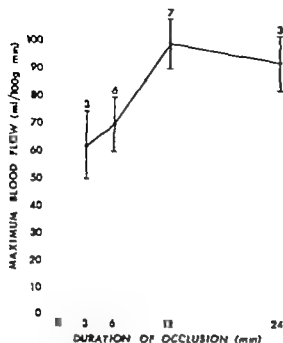


Fig. 3 Maximum blood flow determined from the steepest initial part of the  $^{133}\text{Xe}$  washout curve following release of the cuff is plotted against duration of occlusion.

ty tissue proximal to the area under study is minimized.

The assumptions of a negligible recirculation and a purely blood flow limited washout of tracer seem to be valid for  $^{133}\text{Xe}$  (Sejrsen 1971).

Changes in blood volume in the tissue will affect the cutaneous tissue to blood partition coefficient for  $^{133}\text{Xe}$ . However the influence is less than 3% and can be neglected (Sejrsen 1971). Thus the method seems to be valid under the given conditions.

Excess cumulative blood flow was calculated as  $\ln[q(t)/q(t)_{\text{pre}}]$  (eq. 6) where  $q(t)_{\text{pre}}$  was obtained by retropolating the monoexponential activity curve versus time after the period of reactive hyperemia (see Fig. 7). An alternative way to calculate excess cumulative blood flow is to draw a line in a semi-log plot parallel to the pre-ischemic washout function from  $t_1$  to  $t_2$  starting at  $q(t_1)$ . Excess cumulative blood flow would then equal  $\lambda \ln[q(t_2)/q(t_1)]$  where  $q(t)_{\text{pre}}$  is the ordinate value of this curve at  $t_2$ . This method was not used because the determination of  $t_1$  still depends on retropolation of the final (post-hyperemic) monoexponential part of the washout curve. As already mentioned there was no

significant difference between pre-ischemic and post-ischemic blood flow.

The results obtained are probably not influenced by changes in blood flow through arterio-venous anastomoses for the following reason. There is no arterio-venous anastomoses in the area under study (Hale & Burch 1960). Sejrsen (1971) has shown that the washout rate of  $^{133}\text{Xe}$  in cutaneous tissue is not affected by flow in the larger veins in subcutaneous tissue.

The results obtained are qualitatively similar to those obtained in adipose tissue (Nielsen & Sejrsen 1977) and skeletal muscle (Lindbjerg 1966).

Maximum blood flow increased when duration of ischemia was prolonged from 3 to 12 min (Fig. 3). Further prolongation did not increase maximum blood flow any further, indicating that the arterioles in cutaneous tissue were maximally dilated following 12 min of ischemia. However, as duration of the hyperemic response increased with increasing duration of ischemia up to 24 min (Fig. 4), excess cumulative blood flow still increased when the duration of ischemia was prolonged from 12 to 24 min (Fig. 5). The observation that excess cumulative blood flow did show a further increase when duration of ischemia exceeded that necessary to induce total arteriolar relaxation, signifies that metabolic factors are important in a reactive hyperemia in cutaneous tissue as in other tissue.

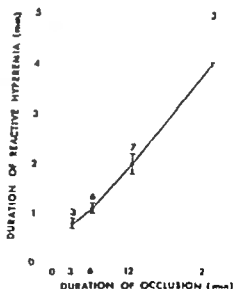


Fig. 4 Duration of reactive hyperemia is plotted against duration of occlusion.

Decrease in oxygen tension might be important. Færchlid *et al.* (1966) observed that reactive hyperemia persisted in the absence of oxygen. There is considerable evidence that oxygen tension influences arteriolar diameters (Düling & Berne 1970, Hellstrand *et al.* 1977, Hellstrand *et al.* 1977, Düling 1972, Hutchins *et al.* 1974). However, increase in carbon dioxide (Kontos & Patterson 1964) and other metabolites might also participate, and so might redistribution of ions across the cell membrane and intracellular membranes.

On the other hand, after induction of ischemia the vascular relaxation might initially be partly elicited by the drop in arterial pressure for the following reasons: Prolonging the duration of vascular occlusion from 10 to 30 s did not cause any increase in excess cumulative blood flow in skeletal muscle (Johnson *et al.* 1976). The hyperemic response was diminished when intra-vascular pressure was maintained by packing the forearm with blood during vascular occlusion (Patterson 1956). The calculated repayment of more than 200% following vascular occlusion up to 120 sec in the myocardium (Coffman & Gregg 1961) is in agreement with this. However, it is not excluded that even after short periods of ischemia repayments exceeding 100% may be elicited by oxygen lack. Myogenic mechanisms seem

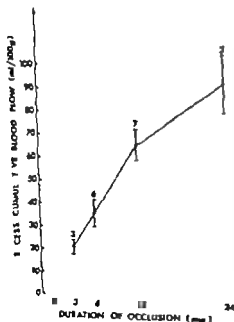


Fig. 5 Excess cumulative blood flow is plotted against duration of occlusion.

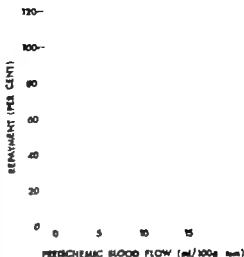


Fig. 6 Repayment is plotted against pre-ischemic blood flow.

quantitatively less important in the present experiments as fractional repayment was maximally 170% (Fig. 6). This might be due to the fact that duration of vascular occlusion was not below 3 min. In skeletal muscle, metabolic factors seem to become important when duration of vascular occlusion exceeds 3 min (Barcroft 1972, Johnson *et al.* 1976). Furthermore, vascular tone in the area under study is highly influenced by sympathetic vasoconstrictor activity which might change the balance between myogenic activity and metabolic factors.

The inverse correlation between repayment and preocclusive blood flow is compatible with the view that normal blood flow in cutaneous tissue is set by factors other than the nutritive demands (e.g. neural impulses governed by temperature regulating centers) or by such factors in addition to those related to the nutritive state of the tissue.

In conclusion, the results of the present study indicate that the reactive hyperemic response in human cutaneous tissue is at least partly due to metabolic factors. However, the combined mechanisms underlying the reactive hyperemic response remain poorly understood.

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# Effects of rubidium caesium strontium barium and lanthanum on ionic currents in myelinated nerve fibres from *Xenopus laevis*.

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ÅRHEM P. Effect of rubidium, caesium, strontium, barium and lanthanum on ionic currents in myelinated nerve fibres from *Xenopus laevis*. Acta Physiol Scand 1980, 108, 7-16. Received 1 March 1979. ISSN 0001-6772. The Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm, Sweden.

A comparative analysis of the effects of externally applied Rb<sup>+</sup>, Cs<sup>+</sup>, Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> on myelinated fibres under potential clamp conditions was made. The ions Cs<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> (belonging to groups 1a, 2a and 3a of period II in the periodic system) were found to affect the K system in specific ways. Ba<sup>2+</sup> and La<sup>3+</sup> reduced the outward K<sup>+</sup> current associated with positive potential steps, the effect of La<sup>3+</sup> being larger than that of Ba<sup>2+</sup>. The effect of Ba<sup>2+</sup> depended on the K<sup>+</sup> concentration. Cs<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> reduced the inward K<sup>+</sup> tail current at repolarisation in solutions with high K<sup>+</sup> concentration. Cs<sup>+</sup> and Ba<sup>2+</sup> caused rectification of the K<sup>+</sup> current, i.e. they reduced the inward tail current proportionally more than the outward steady state current. The effect of Ba<sup>2+</sup> could not be described as a simple reduction of the permeability constant  $\bar{P}$ . Rb<sup>+</sup> was found to affect the time course of the K system by increasing the time constant  $\tau$ . Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> reduced the permeability constant  $\bar{P}$ . These ions also shifted potential dependent parameters in general along the potential axis. The effect of La<sup>3+</sup> was larger than that of Sr<sup>2+</sup> and Ba<sup>2+</sup>. Cs<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> reduced the p-current, Ba<sup>2+</sup> and La<sup>3+</sup> the leak conductance.

**Key words:** Myelinated nerve, ionic currents, metal ions, potential clamp analysis.

In the present investigation the effects on myelinated nerve fibres of the clawed toad *Xenopus laevis* of alkali metal ions (or group 1a ions of the periodic system) Rb<sup>+</sup> and Cs<sup>+</sup>, the alkaline earth metal ions (or group 2a ions) Sr<sup>2+</sup> and Ba<sup>2+</sup> and the transition element ion (or group 3a ion) La<sup>3+</sup> were analysed. The background was the preliminary finding that Cs<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> affected the steady state  $P$  in a rather specific way. Ba<sup>2+</sup> and La<sup>3+</sup> markedly decreased outward  $I_K$  at relatively low concentration. A, C, Ba and La are the first three elements in period 6 of the periodic system, and since it is known that Cs<sup>+</sup> specifically decreases inward  $I_K$  in high [K<sup>+</sup>] free solutions (Dobers & Bergman 1975a, see below) a systematic comparative analysis was performed. The analysis also included the effect of the neighbouring ions Rb<sup>+</sup> and Sr<sup>2+</sup> belonging to period 5. The effect on the ionic currents was studied by the potential clamp technique of Dodge

& Frankenhaeuser (1958) and included effects not only on the potassium and sodium systems, but also on the unspecific p-current (Frankenhaeuser 1962) as well as on the leak conductance.

Earlier investigations have mainly stressed similarities between effects caused by ions within the same chemical group, for instance within group 1a or group 2a. In the present investigation, however, I will stress the similarities between effects caused by ions within the same period, i.e. period II and to some extent period 5.

Among alkali metal ions Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup> and Rb<sup>+</sup> are known to be highly permeable, at least during some phase of the action potential in most excitable membranes. Thus Li<sup>+</sup> and Na<sup>+</sup> have been found to be roughly equally permeant in cephalopod giant axons and amphibian myelinated axons (Baker, Hodgkin & Shaw 1966, Chandler & Meves 1965, Moore et al. 1966, Hille 1977). In potential clamp

Table 1. Composition of solutions

All solutions contained 5 mM Tris-(hydroxymethyl)-amino-methane buffer adjusted to pH 7.2.

Solution	Concentration (mM)			
	Chloride salt of studied ion	NaCl	KCl	CaCl <sub>2</sub>
<i>Reference solutions</i>				
Ringer	-	11.0	2.5	0
Isotonic KCl	-	-	114.5	0
-	-	57.0	57.0	0
<i>Test solutions</i>				
Rb	114.5	-	-	0
	57.0	-	57.0	0
Ca	57.0	-	57.0	0
	10.0	11.0	2.5	0
	10.0	-	114.5	0
Sr <sup>2+</sup> or Ba <sup>2+</sup>	3.4-10.0	11.0	5	0
	3.4-10.0	-	114.5	2.0
La	0.5-1.0	11.0	5	0
	0.5-1.0	-	114.5	0

studies on myelinated fibres from *Rana pipiens* the ratio between the permeability for La and Na at the peak of the early current has been determined to 0.93 (Hille 1977). In a corresponding potential clamp study the permeability ratio for Rb and K at the steady state level of the late current has been determined to 0.93 (Hille 1973).

On the other hand the alkali metal ion Cs is little permeant in cephalopod giant axons and amphibian myelinated axons. Further as mentioned above Cs is known to affect the potassium current in a specific way. Externally applied it reduces the inward current in myelinated fibres from *Rana esculenta* (Dubois & Bergman 1973a). Internally applied it reduces the outward current in *Loligo* giant axon (Armstrong 1973).

Among alkaline earth metal ions Ca, Sr<sup>2+</sup> and Ba<sup>2+</sup> are known to be very little permeant through the membrane of cephalopod giant axons and amphibian myelinated axons. Some other excitable membranes however are highly permeable to these ions during the action potential. This is for instance the case for some mammalian and gastropod nerve cell bodies (Reuter 1973; Hagiwara et al. 1974).

Further as the alkaline earth metal ions are divalent they share what seems to be a common

property of divalent ions in general, namely to shift the potential dependent permeability parameters of excitable membranes at relatively low concentrations. The classical work here is the potential clamp study of the Ca<sup>2+</sup> effects on *Loligo* giant axon (Frankenhaeuser & Hodgkin 1957) in which it was found that Ca<sup>2+</sup> shifts the potential dependent permeability parameters of both the sodium and the potassium system. This effect of Ca<sup>2+</sup> has also been confirmed for amphibian myelinated axons (Frankenhaeuser 1957; Hille 1968; Brismar & Frankenhaeuser 1977). Univalent ions also show such a shift but a smaller one (Mozhayeva & Naumov 1970; Brismar 1973; Hille et al. 1975).

Further a similar shift effect on excitable membranes has been found to be caused by other divalent and trivalent ions at relative low concentrations (Vogel 1974; Hille et al. 1975; Brismar 1979). The effect has been ascribed to a screening of negative surface charges of the membrane. However the situation is complex since there are quantitative differences between the magnitude of the shift caused by different ions in the magnitude of shift of different permeability parameters for one and the same ion and in the magnitude of the shift of the same parameter for different species investigated.

Among transition element ions La is known to shift the potential dependent permeability parameters in myelinated fibres from *Xenopus laevis* in accordance with what is mentioned above (Vogel 1974; Brismar 1979).

## METHODS

Large myelinated nerve fibres from the sciatic nerve of the clawed toad (*Ambystoma* sp.) were used. No attempt was made to distinguish between motor and sensory fibres as made

The effect of the ion in question on the nodal membrane current associated with potential steps were recorded. The membrane potential was controlled by the feed-back system of Dodge & Frankenhaeuser (1958, 1959). The electronic equipment was lightly modified as described earlier (Århem, Frankenhaeuser & Moore 1973). The recording cell and the electrode assembly are held at controlled temperature. The temperature range in the present experiment was 15-22°C.

The experimental procedure was essentially the same as that of Dodge & Frankenhaeuser (1958, 1959). The fibre was mounted in a Perspex cell of the design of Dodge & Frankenhaeuser (1958, 1959); the micromodes on both sides of the node under investigation (N<sub>2</sub>) were cut off at different distances from N<sub>1</sub> in different experiments in order to study the effect of possible voltage changes. The calomel electrodes were connected to the solution pool by 170 mM KCl bridges; the feed-back amplifiers were

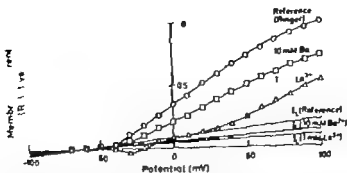


Fig. 1. The effect of Ba<sup>2+</sup> (10 mM) and La<sup>3+</sup> (1 mM) on the steady state current-potential relation in test solutions with 2.5 mM K. The net membrane current as measured 11 ms after the onset of the test step,  $I$ , is leak current,  $I_L$ , is obtained by subtracting  $I_L$  from the steady state current. Note the inward current at low potential steps for the run with La<sup>3+</sup>. This is usually an  $I_{Na}$  since the sodium system is not fully inactivated at 6 ms and low test steps. La<sup>3+</sup> was found to prolong the  $\tau$ . Holding potential was -90 mV. At 19 °C Temperature 5 °C.

adjusted and turned on. The membrane potential was stepped in steps, and the associated currents were measured. Measurements were made with test solutions applied to the node under investigation.

The sodium peak permeability associated with potential step is calculated according to Dodge & Franzblau (1959) from the peak sodium current, using the constant field equation. The expression for the single ionic current which can be calculated from the Nernst-Planck equation if the electric field inside the membrane is constant (Goldman 1943; Hodgson & Katz 1949).

The steady state potential permeability associated with potential step was calculated correspondingly by the constant field equation from either (1) the steady state current-potential relation ( $I_{ss}$  vs  $U$  relation) in solutions of Ringer type (see next section), (2) the  $(I_{ss} - U_{ss})$  relation in solutions of isotonic KCl type or (3) the instantaneous  $I$  at repolarisations at solutions of isotonic KCl type (Franzblau 1962).

All the currents are given in relative units because of the uncertainty of the calibration factor and consequently the permeabilities also are given in relative units.

**Solutions.** The composition of reference and test solutions are given in Table 1.

When analysing the experiments and comparing the results some special points have to be taken into account:

(1) LaCl<sub>3</sub> is hygroscopic and therefore too high concentrations may be given in the text for some experiments. Further the slow acidification of LaCl<sub>3</sub> solution must be taken into account. Thus only fresh solutions were used in the experiments.

(2) The activity coefficient for divalent and trivalent ions at the ionic strength used in these experiments deviates considerably from 1.0. A theoretical value for the activity coefficient of an ionic species (2) that was used in comparing the efficiency of divalent and trivalent ions is given by the equation

$$\log f = -0.51z^2\sqrt{I}$$

where  $z$  is the ionic strength of the solution and  $z$  the valency of the ion (Morris 1968).

(3) Many buffer solutions form metal-buffer complexes. The metal/buffer binding constants may be considerable. The only binding constant that may be of importance here is that between Tris buffer and La<sup>3+</sup> because La<sup>3+</sup> is used in low concentrations relative to the Tris concentration. The value of this was not found. However the binding constants for Tris/buffer and Mg<sup>2+</sup>, Ca<sup>2+</sup> and Mn<sup>2+</sup> are known to be negligible (Sober 1968).

**Conventions.** Membrane potential (denoted  $U_m$ ) is given as inside potential minus outside potential. Consequently outward current is positive. The concepts negative and positive polarisation are used instead of the ambiguous concepts hyperpolarisation and depolarisation.

## RESULTS

### Effects on the potassium system

**Period 6 ions Cs, Ba<sup>2+</sup> and La<sup>3+</sup>.** The first step was to confirm that Cs in high concentrations (>10 mM) in test solutions containing high [K] reduced the inward  $I$  associated with the repolarisation at the end of a positive potential step, without affecting the outward  $I$  associated with the positive potential step.

The next step was to analyse the effects of Ba<sup>2+</sup> and La<sup>3+</sup> in more detail. The effect on the steady state current-potential relation is shown in Fig. 1. The main effect of Ba<sup>2+</sup> on the potassium system in a low [K] test solution was a general decrease of  $I$  associated with a positive potential step without any marked effect on the time course of  $I$ . The onset of this effect was so rapid that it was impossible in the present experiments to separate the time



Table 2. Effects of  $Ba^{2+}$ ,  $La^{3+}$  and  $Sr^{2+}$  on permeability properties

Effects on  $P$ ,  $\bar{P}_{Na}$  and  $g_i$  measured as the ratio between the value in test solution and in reference solution. Calculations of  $P$  were made in solutions containing 5.5 mM respectively 114.5 mM  $K^+$  (from measurements of  $v_{Na}$  and steady  $I_K$  respectively inward tail  $I$ ).  $\Delta U$  is the shift along the potential axis of sodium activation curve (measured as difference between the potential at which peak  $\bar{P}_{Na}$  reaches half its maximum value in Ringer solution and in test solution).

Tested ion	Axon	Temp (°C)	[ $K^+$ ] = 5 mM	[ $K^+$ ] = 114.5 mM Measurement from		$\bar{P}_{Na}$	$\Delta U$ (mV)	$g_i$	
				Steady state $I$	Tail $I$			[ $K^+$ ] = 5 mM	[ $K^+$ ] = 114.5 mM
[ $Ba^{2+}$ ] = 10 mM	5	-2	0.67	-	-	0.85	18	1.0	-
	17	5	0.77	-	-	0.44	19	1.0	-
	18	5	0.63	-	-	0.9	17	1.0	-
	19	5	0.74	-	-	0.58	1	0.7	-
	30	20	-	1.0	0.43	-	-	-	0.9
	31	20	-	0.96	0.30	-	-	-	-
	3	20	-	0.93	0.45	-	-	-	-
	33	20	-	0.81	0.40	-	-	-	-
	Mean		0.70	0.9	0.39	0.59	16	0.9	0.9
[ $La^{3+}$ ] = 1 mM	13	1	0.47	-	-	0.43	17	0.6	-
	17	5	0.54	-	-	0.44	19	0.6	-
	19	5	0.53	-	-	0.58	16	0.9	-
	30	20	-	0.47	0.9	-	-	-	0.9
	Mean		0.51	0.47	0.9	0.48	18	0.9	0.9
[ $Sr^{2+}$ ] = 10 mM	3	-	1.0	-	-	0.77	17	1.0	-
	5	-	1.0	-	-	0.81	18	1.0	-
	31	20	-	1.0	0.70	-	-	-	1.0
	Mean		1.0	1.0	0.70	0.76	18	1.0	1.0

course of the effect and the time course of the concentration change. The effect was further fully reversible. So far the effect is consistent with the idea of a decrease of the permeability constant  $\bar{P}_K$  (see Frankenhaeuser 1962). The decrease of the maximum value of the steady state  $\bar{P}_K$  for 10 mM  $Ba^{2+}$  in 4 fibres is given in Table 2. The average decrease was 30%.

In order to analyse the effect on inward  $I$  (of the  $Cs^+$  effect) and on the potassium activation curve (steady state  $P - U_{Na}$  curve) experiments were performed with high [ $K^+$ ] test solutions. The motivation for this procedure was that the driving force for  $K^+$  in the potential region where  $P$  is turned on is larger in a high [ $K^+$ ] solution and  $I$  is consequently larger.

It was found that  $Ba^{2+}$  like  $Cs^+$  caused an inward rectification: the inward  $I_K$  associated with the repolarisation at the end of a positive step was reduced proportionally more than the steady state outward  $I$  during the positive potential step. This is clearly seen in Fig. 2 and Fig. 3. In Fig. 2 the effect

on the steady state current (ordinate 1 is obtained by subtracting  $I_i$  from the steady state current) is associated with a potential step (abscissae) while Fig. 3 is a corresponding plot of the value of the peak inward current associated with the repolarisation (ordinates) from a preceding conditioning step (abscissae). The rectification of the  $I_K$  is inconsistent with the description of the  $Ba^{2+}$  effect as only decreasing the permeability constant  $\bar{P}_K$ . The effect seemed independent of temperature in the range 5–22°C. Further it seemed independent of whether the fibre was cut at the middle of the internode or at neighbouring node.

The effect of 10 mM  $Ba^{2+}$  on the maximum value of  $P$  in 4 fibres is shown in Table 2. The  $P$  was calculated from (i) the steady state  $I$  associated with positive potential steps or (ii) the peak value of the inward  $I$  tail associated with repolarisation. The average decrease caused by the [ $Ba^{2+}$ ] in the two cases was 8% and 61% respectively.

The rectification is evident from these calculations. Another aspect of the  $Ba^{2+}$  effect is further

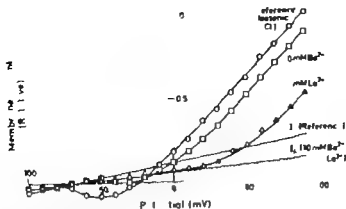


Fig. 2 The effect of Ba<sup>2+</sup> (10 mM) and La<sup>3+</sup> (1 mM) on the steady state current-potential relation in test solutions with 114.5 mM K. The net membrane current was measured at 5 ms after the onset of the test step. The effect on the leak current ( $I_L$ ) is shown. Holding potential as 100 mV. Axon 30. Temperature 20°C.

from Table 1. A comparison between the values given for the experiments in low respectively high [K<sup>+</sup>] test solutions shows that Ba<sup>2+</sup> caused a more pronounced decrease in the steady state  $I_L$  between external [K<sup>+</sup>] as compared to high. This might indicate an interaction between K<sup>+</sup> and Ba<sup>2+</sup> with respect to the effects on the potassium system. As expected Ba<sup>2+</sup> was found to shift the potassium activation curve (steady state P-U curve) along the potential axis. This was analysed in experiments with high [K<sup>+</sup>] test solutions in order to obtain an optimal resolution. The shift, caused by La<sup>3+</sup> corresponded to that of Mg<sup>2+</sup> and Ca<sup>2+</sup> which also are group 2 ions. However the effect of Ba<sup>2+</sup> was more complex, since Ba<sup>2+</sup> also caused the rectification of  $I_L$ . The shift of P along the potential axis caused by 10 mM Ba<sup>2+</sup> was about 35 mV when estimated from  $I_L$  tails in high [K<sup>+</sup>] test solutions.

Ba<sup>2+</sup> was further found to affect the kinetics of the potassium system. At high potentials ( $U \geq 50$  mV) the time constant  $\tau$  was increased slightly while at low potentials ( $U \leq 70$  mV) as estimated from  $I_L$  tail  $\tau$  was decreased. The increase of  $\tau$  at high potentials is equivalent to a decrease of the rate constant  $\alpha_\infty$  since at these potentials  $\tau$  is proportional to  $1/\alpha_\infty$ . Likewise the decrease of  $\tau$  at low potentials is equivalent to an increase of  $\beta_\infty$  since at these potentials  $\tau$  is proportional to  $1/\beta_\infty$ . The effect of Ba<sup>2+</sup> was larger on  $\beta_\infty$  than on  $\alpha_\infty$ .

Concerning the effect of La<sup>3+</sup> on the potassium system the findings of Vogel (1974) were confirmed that La<sup>3+</sup> decreased the maximum P. In contrast to the effect of Ba<sup>2+</sup> the effect of La<sup>3+</sup> was only partially reversible. The effect on the steady state current-potential relation is shown in Fig. 1. The decrease of the steady state P caused by 1 mM La<sup>3+</sup>

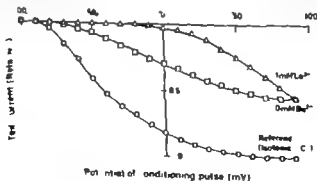


Fig. 3 The effect of Ba<sup>2+</sup> (10 mM) and La<sup>3+</sup> (1 mM) on the current tail at repolarization from potential step to 100 mV in test solutions with 114.5 mM K. Current plotted against the potential of the preceding step. The same axon as in Fig. 2.

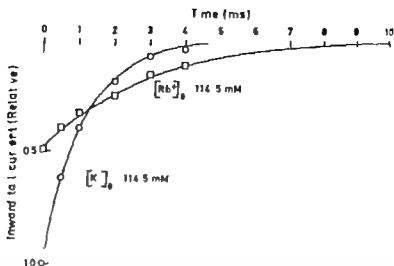


Fig. 4. The effect of Rb on  $\tau$ . The tail current at repolarisation to  $-110$  mV from a potential step of  $70$  mV in  $114.5$  mM Rb solution and in  $114.5$  mM K solution. Smooth curves are simple exponential curves fitted to the data. Time constants used were  $1.1$  ms and  $2.8$  ms respectively. Axon 4. Temperature  $22^\circ\text{C}$ .

is presented in Table 2. The average decrease was 49% which is less than the corresponding decrease described by Vogel (1974). The effect on  $P_h$  seemed to be independent of the temperature in the range  $5$ – $27^\circ\text{C}$ .

In order to find out whether or not  $\text{La}^{3+}$  caused a rectification similar to that caused by Cs and Ba, the effect was further analysed in high  $[\text{K}]$  test solutions. Calculations based on the data presented in Fig. 2 and Fig. 3 indicate an absence of a rectification of the kind caused by Ba.  $\text{La}^{3+}$  caused an equally large percentage decrease of the steady state  $I_k$  and the  $I_k$  tail. Further measurements indicated that  $\text{La}^{3+}$  decreased the permeability constant  $P_h$ .  $\text{La}^{3+}$  shifted the potassium activation curve (steady state  $P_h$ – $U_m$  curve) along the potential axis similarly as the group 2 ions  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ . An analysis in high  $[\text{K}]$  test solutions confirmed the results of Vogel (1974) who made measurements in low  $[\text{K}]$  solutions. The shift is clearly seen in Fig. 2 and Fig. 3 where the effect is shown on the current potential relation in two types of measurements. It should be noted however that the steady state  $I_k$  was not reached in the experiment with  $\text{La}^{3+}$  shown in the figures. The magnitude of the shift was large, in agreement with the earlier measurements by Vogel (1974).

$\text{La}^{3+}$  was further found to affect the kinetics of the potassium system. Large potential steps in increased  $\tau$  independent of whether test solutions with low or with high  $[\text{K}]$  were used.  $1$  mM  $\text{La}^{3+}$

increased  $\tau$  by a factor of 3 or 4 in the potential range  $50$  to  $100$  mV which indicates that the  $\alpha$  constant decreased. At low potentials ( $U > 50$  mV)  $\tau$  decreased by a factor of 10–4. This was studied in experiments with high  $[\text{K}]$  test solutions (inward  $I_k$  tails). This indicates an increase of the rate constant  $\beta_h$ . These findings of the effect on  $\tau$  are consistent with the hypothesis that  $\text{La}^{3+}$  shifts the potential dependent parameters along the potential axis.

**Period 5 ions Rb and  $\text{Sr}^{2+}$**  The effects of the period 5 ions Rb and  $\text{Sr}^{2+}$  on the potassium system were also analysed. The well known earlier finding (Hille 1973) was confirmed that Rb can carry the delayed membrane current. This was analysed in experiments with an isotonic test solution with high  $[\text{Rb}]$  and no  $[\text{K}]$ . The outward current associated with large positive potential steps was unchanged while the inward tail current thus here carried by Rb at the end of the potential step decreased about 50%. This is consistent with the view that Rb is about half as permeant as K through the potassium transport system. This is in contrast to the findings in *Rana pipiens* where the corresponding Rb/K permeability ratio is about 0.9 (Hille 1973).

However, when the tail current in a test solution containing  $57$  mM Rb and  $57$  mM K was compared with the corresponding current in an isotonic KCl solution the decrease was larger than expected from the measurements in isotonic RbCl solution.

Rb was found to affect the kinetics also of the

sum system which was seen in experiments solutions containing high [Rb<sup>+</sup>] and low [K<sup>+</sup>]. The course of the current tail (thus carried by Rb<sup>+</sup>) was slow compared to the current of the tail in high [K<sup>+</sup>] solution. In the solution with 114.5 mM Rb<sup>+</sup> and no K<sup>+</sup> the time constant was about twice as large as in isotonic solution at corresponding steps (Fig. 4). No effect by Rb<sup>+</sup> was noted on the time course of the sum permeability change associated with positive potential steps. Thus Rb<sup>+</sup> affected only at potentials close to the resting potential or more negative potentials. Since  $\omega = 1/(A_m + \beta A_m)$   $A_m$  is negligibly small at a membrane potential as negative than resting potential it is evident  $A_m$  is responsible for the effect of Rb<sup>+</sup> on the potential region.

The effect of Sr<sup>2+</sup> on the potassium system was tested as with Mg<sup>2+</sup> and Ca<sup>2+</sup>. The main effect of Sr<sup>2+</sup> was a shift of the potassium activation curve (steady state  $P - U_m$  curve) along the potential axis. The shift was for the same reasons as those given above analysed in experiments where the test solution contained high [K<sup>+</sup>]. The magnitude of the shift of the potassium activation curve caused by 10 mM Sr<sup>2+</sup> was smaller than that caused by 10 mM Ba<sup>2+</sup>.

In contrast to the effect of Ba<sup>2+</sup> Sr<sup>2+</sup> caused no or only a small decrease of the steady state  $I_{Na}$  associated with large positive potential steps. On the other hand in high [K<sup>+</sup>] solutions was found to increase the inward  $I_{Na}$ . In one experiment with 10 mM Sr<sup>2+</sup> a decrease of 30% was observed (Table 1). The effects of Sr<sup>2+</sup> were fast and fully reversible.

Effects on the sodium system

Further potential clamp in saturation on amphibian gill cells concerning the effects of Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> on the Na<sup>+</sup> system (Vogel 1974; Hille et al. 1975 and Bergman 1979) were confirmed in the present investigation.

(i) Thus Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> decreased the maximum value of peak  $P_{Na}$ . As this was seen in experiments where the holding potential was -90 mV or more negative and the inactivation consequently was kept at negligibly low level, it was concluded that this decrease was caused by a decrease of the permeability constant  $P_{Na}$ . However, the cautionness in this interpretation is necessary. Thus a change was noted in the shape of the curve relating sodium activation to potential the

slope of this curve was less in test solutions containing Sr<sup>2+</sup>, Ba<sup>2+</sup> or La<sup>3+</sup> than in the reference Ringer solution. Another complication in the experiments was that the effect of La<sup>3+</sup> on the sodium system, as in the case of the potassium system, was only partially reversible. The results of the present investigation are consistent with the findings from earlier investigations that the effect of La<sup>3+</sup> on the peak  $P_{Na}$  at large positive steps is larger than that of Sr<sup>2+</sup> and Ba<sup>2+</sup>. The decrease of peak  $P_{Na}$  was slightly less in the present experiments than that reported by Vogel (1974). It is important here to note the quantitative similarity between the Sr<sup>2+</sup> and Ba<sup>2+</sup> effects on the sodium system in contrast to the effects on the potassium system.

(ii) Further Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> shifted the sodium activation curve along the potential axis in positive direction. The present findings are consistent with earlier findings of the efficiency sequence concerning the magnitude of the shift of the sodium activation curve. Thus

$$La^{3+} > Ca^{2+} > Ba^{2+} \approx Sr^{2+}$$

The shift caused by La<sup>3+</sup> was slightly smaller in the present experiments than that reported by Vogel (1974) while the shifts by Sr<sup>2+</sup> and Ba<sup>2+</sup> were slightly larger than those reported by Hille et al. (1975).

(iii) Concerning the time dependent parameters a general slow down was observed of the kinetics of the sodium system by Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup>. This is in accordance with the hypothesis that these ions cause a general shift of the potential dependent parameters. However preliminary calculations showed that the effect of La<sup>3+</sup> on  $\tau_{Na}$  was larger than expected from the potential shift per se as calculated above from the sodium activation curve.

#### Effect on p-current and leak conductance

Some observations concerning the effects of the ions in question on the non-specific delayed current (the p-current,  $I_p$ ) and the leak conductance ( $g_L$ ) were also made in the present investigation. Thus it was found that the period 6 ions, Cs<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> all systematically decreased  $I_p$ . The findings of the effect of Cs<sup>+</sup> were in agreement with the observations of Dubois & Bergman (1975). Calcium affected  $I_p$  at lower concentrations (10 mM) than the inward  $I_{Na}$  in a high [K<sup>+</sup>] solution. No effect of the period 5 ions Rb<sup>+</sup> and Sr<sup>2+</sup> on  $I_p$  was noted.

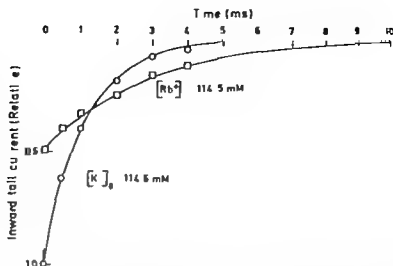


Fig. 4. The effect of Rb on the tail current at repolarisation to  $-110$  mV from a potential step of  $70$  mV in  $114.5$  mM Rb solution and in  $114.5$  mM K solution. Smooth curves are simple exponential curves fitted to the data. Time constants used were  $1.1$  ms and  $2.8$  ms respectively. Axon 4. Temperature  $22^\circ\text{C}$ .

is presented in Table 2. The average decrease was 49% which is less than the corresponding decrease described by Vogel (1974). The effect on  $P$  seemed to be independent of the temperature in the range  $5$ – $22^\circ\text{C}$ .

In order to find out whether or not  $\text{La}^{3+}$  caused a rectification similar to that caused by  $\text{Cs}$  and  $\text{Ba}^{2+}$  the effect was further analysed in high  $[\text{K}]$  test solutions. Calculations based on the data presented in Fig. 2 and Fig. 3 indicate an absence of a rectification of the kind caused by  $\text{Ba}$ .  $\text{La}^{3+}$  caused an equally large percentage decrease of the steady state  $I$  and the  $I$  tail. Further measurements indicated that  $\text{La}^{3+}$  decreased the permeability constant  $\bar{P}$ .  $\text{La}^{3+}$  shifted the potassium activation curve (steady state  $P-U_m$  curve) along the potential axis similarly as the group 2 ions  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ . An analysis in high  $[\text{K}]$  test solutions confirmed the results of Vogel (1974) who made measurements in low  $[\text{K}]$  solutions. The shift is clearly seen in Fig. 2 and Fig. 3 where the effect is shown on the current-potential relation in two types of measurements. It should be noted however that the steady state  $I$  was not reached in the experiment with  $\text{La}^{3+}$  shown in the figures. The magnitude of the shift was large in agreement with the earlier measurements by Vogel (1974).

$\text{La}^{3+}$  was further found to affect the kinetics of the potassium system. Large potential steps increased  $\tau$  independent of whether test solutions with low or with high  $[\text{K}]$  were used.  $1$  mM  $\text{La}^{3+}$

increased  $\tau$  by a factor of 3 or 4 in the potential range  $50$  to  $100$  mV which indicates that the constant  $\alpha$  decreased. At low potentials ( $U > 50$  mV)  $\tau$  decreased by a factor of 2 to 4. This is consistent with findings in experiments with high  $[\text{K}]$  test solutions (inward  $I_h$  tails). This indicates an increase in the rate constant  $\beta_h$ . These findings of the effect on  $\tau$  are consistent with the hypothesis that  $\text{La}^{3+}$  shifts the potential dependent parameters along the potential axis.

**Period 5 ions Rb and  $\text{Sr}^{2+}$**  The effects of the period 5 ions Rb and  $\text{Sr}^{2+}$  on the potassium system were also analysed. The well known earlier finding (Hille 1973) was confirmed that Rb can carry the delayed membrane current. This was analysed in experiments with an isotonic test solution with high  $[\text{Rb}]$  and no  $[\text{K}]$ . The outward current associated with large positive potential steps was unchanged, while the inward tail current thus here carried by Rb at the end of the potential step decreased about 50%. This is consistent with the view that Rb is about half as permeant as K through the potassium transport system. This is in contrast to the findings in *Rana pipiens* where the corresponding Rb/K permeability ratio is about 0.9 (Hille 1973).

However, when the tail current in a test solution containing  $57$  mM Rb and  $57$  mM K was compared with the corresponding current in an isotonic KCl solution the decrease was larger than expected from the measurements in isotonic RbCl solution.

Rb was found to affect the kinetics also of the

sum system which was seen in experiments solutions containing high [Rb<sup>+</sup>] and low [K<sup>+</sup>], true course of the current tail (thus carried by Rb<sup>+</sup>) was slow compared to the corresponding current tail in high [K<sup>+</sup>] solution. In the solution with 114.5 mM Rb<sup>+</sup> and no K<sup>+</sup>, the time tail was about twice as large as  $\tau$  in isotonic solution in corresponding steps (Fig. 4). No effect by Rb<sup>+</sup> was noted on the time course of the steady permeability change associated with positive potential steps. Thus Rb<sup>+</sup> affected  $\tau$  only at potential close to the resting potential or more negative potentials. Since  $\tau = 1/(\alpha_a + \beta_a)$ ,  $\alpha_a$  is negligibly small at a membrane potential as negative than resting potential it is evident that  $\beta_a$  is responsible for the effect of Rb<sup>+</sup> on  $\tau$  in potential region.

The effect of Sr<sup>2+</sup> on the potassium system was typical. As with Mg<sup>2+</sup> and Ca<sup>2+</sup>, the main effect of Sr<sup>2+</sup> was a shift of the potassium activation curve (steady state  $P \sim U$  curve) along the potential axis. This was for the same reasons as those given above analysed in experiments where the test solution contained high [K<sup>+</sup>]. The magnitude of the shift of the potassium activation curve caused by 10 mM Sr<sup>2+</sup> was smaller than that caused by 10 mM

La<sup>3+</sup>. In contrast to the effect of Ba<sup>2+</sup>, Sr<sup>2+</sup> caused no or only a small decrease of the steady state  $I$  associated with large positive potential steps. On the other hand, in high [K<sup>+</sup>] solutions was found to increase the inward  $I$  tail. In one experiment with small Sr<sup>2+</sup> a decrease of 30% was observed (Table 1). The effects of Sr<sup>2+</sup> were fast and fully reversible.

#### Effects on the sodium system

Earlier potential clamp investigations on amphibian nerves concerning the effect of Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> on the Na<sup>+</sup> system (Vogel 1974; Hille et al. 1975 and present 1979) were confirmed in the present investigation.

(i) Thus Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> decreased the activation slope of peak  $P_{Na}$ . As this was seen in experiment where the holding potential was -90 mV or more negative and the inactivation consequently kept at negligibly low level it was concluded that this decrease was caused by a decrease of the permeability constant  $P_{Na}$ . However some cautionness in this interpretation is necessary. Thus change was noted in the shape of the sodium relating sodium activation to potential, the

slope of this curve was less in test solutions containing Sr<sup>2+</sup>, Ba<sup>2+</sup> or La<sup>3+</sup> than in the reference Ringer solution. Another complication in the experiments was that the effect of La<sup>3+</sup> on the sodium system as in the case of the potassium system, was only partially reversible. The results of the present investigation are consistent with the findings from earlier investigations that the effect of La<sup>3+</sup> on the peak  $P_{Na}$  at large positive steps is larger than that of Sr<sup>2+</sup> and Ba<sup>2+</sup>. The decrease of peak  $P_{Na}$  was slightly less in the present experiments than that reported by Vogel (1974). It is important here to note the quantitative similarity between the Sr<sup>2+</sup> and Ba<sup>2+</sup> effects on the sodium system in contrast to the effects on the potassium system.

(ii) Further Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> shifted the sodium activation curve along the potential axis in positive direction. The present findings are consistent with earlier findings of the efficiency sequence concerning the magnitude of the shift of the sodium activation curve. Thus

$$La^{3+} > Ca^{2+} > Ba^{2+} \approx Sr^{2+}$$

The shift caused by La<sup>3+</sup> was slightly smaller in the present experiments than that reported by Vogel (1974) while the shifts by Sr<sup>2+</sup> and Ba<sup>2+</sup> were slightly larger than those reported by Hille et al. (1975).

(iii) Concerning the time dependent parameters a general slow down was observed of the kinetics of the sodium system by Sr<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup>. This is in accordance with the hypothesis that these ions cause a general shift of the potential dependent parameters. However preliminary calculations showed that the effect of La<sup>3+</sup> on  $\tau$  was larger than expected from the potential shift per se as calculated above from the sodium activation curve.

#### Effects on p-current and leak conductance

Some observations concerning the effects of the ions in question on the non-specific delayed current (the p-current  $I_p$ ) and the leak conductance ( $g_L$ ) were also made in the present investigation. Thus it was found that the period 3 ions Ca<sup>2+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> all systematically decreased  $I_p$ . The findings of the effect of Ca<sup>2+</sup> were in agreement with the observations of Dubois & Bergman (1975a). Calcium affected  $I_p$  at lower concentrations (10 mM) than the inward  $I$  in a high [K<sup>+</sup>] solution. No effect of the period 5 ions Rb<sup>+</sup> and Sr<sup>2+</sup> on  $I_p$  was noted.

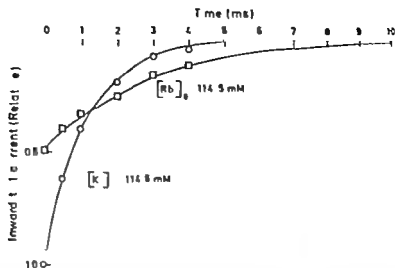


Fig. 4. The effect of Rb on  $\tau$ . The tail current at repolarization to  $-110$  mV from a potential step of  $70$  mV in  $114.5$  mM Rb solution and in  $114.5$  mM K solution. Smooth curves are simple exponential curves fitted to the data. Time constants used were  $1.1$  ms and  $0.8$  ms respectively. Axon 4. Temperature  $22^\circ\text{C}$ .

is presented in Table 2. The average decrease was  $49\%$  which is less than the corresponding decrease described by Vogel (1974). The effect on  $P_A$  seemed to be independent of the temperature in the range  $5$ – $22^\circ\text{C}$ .

In order to find out whether or not  $\text{La}^{3+}$  caused a rectification similar to that caused by Cs and  $\text{Ba}^{2+}$ , the effect was further analysed in high  $[\text{K}]_o$  test solutions. Calculations based on the data presented in Fig. 2 and Fig. 3 indicate an absence of a rectification of the kind caused by  $\text{Ba}^{2+}$ .  $\text{La}^{3+}$  caused an equally large percentage decrease of the steady state  $I$  and the  $I_{\text{tail}}$ . Further measurements indicated that  $\text{La}^{3+}$  decreased the permeability constant  $\tilde{P}$ .  $\text{La}^{3+}$  shifted the potassium activation curve (steady state  $P_A$ – $U_m$  curve) along the potential axis similarly as the group 2 cations  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ . An analysis in high  $[\text{K}]_o$  test solutions confirmed the results of Vogel (1974) who made measurements in low  $[\text{K}]_o$  solutions. The shift is clearly seen in Fig. 2 and Fig. 3 where the effect is shown on the current-potential relation in two types of measurements. It should be noted however that the steady state  $I$  was not reached in the experiment with  $\text{La}^{3+}$  shown in the figures. The magnitude of the shift was large, in agreement with the earlier measurements by Vogel (1974).

$\text{La}^{3+}$  was further found to affect the kinetics of the potassium system. Large potential steps increased  $\tau$  independent of whether test solutions with low or with high  $[\text{K}]_o$  were used.  $1$  mM  $\text{La}^{3+}$

increased  $\tau$  by a factor of 3 or 4 in the potential range  $50$  to  $100$  mV which indicates that the constant  $\alpha$  decreased. At low potentials ( $U > 5$  mV)  $\tau$  decreased by a factor of 2 to 4. This was studied in experiments with high  $[\text{K}]_o$  test solutions (inward  $I_{\text{tail}}$ ). This indicates an increase of the rate constant  $\beta_m$ . These findings of the effect on  $\tau$  are consistent with the hypothesis that  $\text{La}^{3+}$  shifts the potential dependent parameters along the potential axis.

**Period 5 ions Rb and  $\text{Sr}^{2+}$**  The effects of the period 5 ions Rb and  $\text{Sr}^{2+}$  on the potassium system were also analysed. The well known earlier finding (Hille 1973) was confirmed that Rb can carry its delayed membrane current. This was analysed in experiments with an isotonic test solution with  $[\text{Rb}]_o$  and no  $[\text{K}]_o$ . The outward current associated with large positive potential steps was unchanged while the inward tail current thus here carried by Rb at the end of the potential step decreased about  $50\%$ . This is consistent with the view that Rb is about half as permeant as K through the potassium transport system. This is in contrast to the finding in *Rana pipiens* where the corresponding Rb/K permeability ratio is about  $0.9$  (Hille 1973).

However, when the tail current in a test solution containing  $57$  mM Rb and  $57$  mM K was compared with the corresponding current in an isotonic KCl solution the decrease was larger than expected from the measurements in isotonic RbCl solution.

Rb was found to affect the kinetics also of the

e in low [K<sub>i</sub>], than in high [K<sub>i</sub>]. Dubois & Bergman (1977) have proposed a model consisting of pores with a wider external mouth extending at halfways into the membrane. The width of external part of the pore was postulated to be large enough to let C<sup>+</sup> and K<sup>+</sup> move in a single file turn. Further they proposed a site with high K<sup>+</sup> affinity in the pore, the location being about at the same distance from the external as from internal ends of the pore. The K<sup>+</sup> site had to be occupied in order to keep the pore open. The outward I<sub>1</sub> associated with large potential steps was found to increase under certain conditions when the external solution contained no K<sup>+</sup>. Dubois & Bergman concluded from their experiments that, within the lines of the presented model Cs<sup>+</sup> could replace K<sup>+</sup> at the site. The Ba<sup>2+</sup> interaction with K<sup>+</sup> found in present investigation might be interpreted as a competition with the postulated K<sup>+</sup> site. Further Rb<sup>+</sup> is also assumed to react with the K<sup>+</sup> site. Test solutions containing no K<sup>+</sup> and 114.5 mM Rb<sup>+</sup> did not decrease the outward I<sub>1</sub>.

#### Tests on the sodium system

The effect of the sodium action curve by the addition of earth metal ion, and by La<sup>3+</sup> was found to be similar to that reported from *Rana pipiens* (Hille et al 1975). The efficiency sequence was the same. Our findings support the hypothesis of screening and binding to fixed surface charges in the membrane based on the Gossy-Chapman-Stern double layer model (Grahame 1947; Gilbert & Brenstrom 1969; Borsos 1973; Hille et al 1975). The efficiency sequence for *Rana pipiens* and *Xenopus laevis* differs in one respect from the corresponding sequence for *Hemoris americana* (Brenstrom & Goldman 1968) in the latter case Ba<sup>2+</sup> is larger than Cs<sup>+</sup>.

#### Effects on p-currents and leak conductance

The period A ions C<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup> all decreased the I<sub>1</sub> tail and the p-current tail (I<sub>p</sub>) at repolarization. The effect of C<sup>+</sup> was in agreement with the results from *Rana esculenta* (Dubois & Bergman 1975).

It is known from several investigations that many substances both affect I<sub>1</sub> and affect I<sub>p</sub>. Examples are Cs<sup>+</sup> (internally applied Dubois & Bergman 1975b), tetraethylammonium ions (TEA both ex-

ternally and internally applied Koppenhöfer 1967; Koppenhöfer & Vogel 1969), bis-(p-chlorophenyl)-acetic acid (DDA affects the time course Arhem & Frankenhaeuser 1974), aniline (Dubois & Bergman 1975b). However the situation is complex. In preliminary experiments I found that the barbiturate methohexital increased the outward I<sub>1</sub> associated with large potential steps but decreased I<sub>1</sub>. It should also be noted that different amphibian species seem to differ with respect to the magnitude of I<sub>1</sub> (Armstrong & Hille 1977).

In summary this might seem to support the hypothesis that the I<sub>1</sub> tail is caused by an accumulation of K<sup>+</sup> in a perinodal space. However the hypothesis of an aqueous space bounded by a perinodal barrier (Dubois & Bergman 1975b) is inconsistent with several experimental findings. Thus the time course of the I<sub>1</sub> tail current is voltage dependent and the magnitude of I<sub>1</sub> measured at one potential reaches a limiting maximum value even when the preceding steady state I<sub>1</sub> is increased (Frankenhaeuser 1963). The simple idea that an accumulation of K<sup>+</sup> in a perinodal space is the basis for the I<sub>1</sub> tail must thus be rejected as it was originally rejected by Frankenhaeuser (1963).

Concerning the leak conductance (g<sub>L</sub>) the selectivity order for the alkali ions found in the present investigation differed in one respect from that reported from fibres in *Rana pipiens* (Hille 1977). Rb<sup>+</sup> was more permeant than Cs<sup>+</sup> in *Xenopus laevis* fibres. It should be pointed out, that no precaution to eliminate a possible contribution from the time and voltage dependent potassium system by TEA was taken in the present investigation as was done by Hille in the investigation of the *Rana pipiens* fibres. In conclusion the results on g<sub>L</sub> further stress the fact that the resting potential is not explained by simple electrodiffusion and the constant field assumption (Arhem et al 1974; Jack 1976). The increase of g<sub>L</sub> in a solution containing 114.5 mM K<sup>+</sup> relative to g<sub>L</sub> in Ringer solution was found to be about 30%. If electrodiffusion and the constant field conditions are assumed and further that P<sub>Na</sub>/P<sub>K</sub> is 0.05 which would be required for resting potential of -70 mV, then the predicted increase in g<sub>L</sub> would be about 370%.

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Table 3. Some physical and chemical properties of the alkali metals, the alkaline earth metals and transition element lanthanum.

$H_h$  is standard enthalpy change on hydration at 298 K.  $N$  is electronegativity index. Data from Alfred (1961) and Reddy (1970) and Harrison (1977).

Group	Ion	Ionic radius (pm)	$H_h$ (kJ mol <sup>-1</sup> )	Mean hydration number (from enthalpy measurements)	$N$
1a	Li	68	-499.1	5	0.98
	Na	98	-389.9	4	0.91
	K	131	-305.4	3	0.82
	Rb	148	-280.7	3	0.8
	Cs	167	-47.7	-	0.79
2a	Be	30	-	-	1.57
	Mg <sup>2+</sup>	65	-1891	13	1.31
	Ca	94	-1361.0	10	1.00
	Sr <sup>2+</sup>	110	-1413.0	-	0.95
	Ba <sup>2+</sup>	134	-1272.8	-	0.89
3d	La	115	-	-	1.10

The leak conductance was calculated in the normal way from measurements of the leak current associated with a negative potential step. In order to keep the permeability of the potential and time dependent sodium and potassium system at a stable level, the holding potential was kept at -90 mV or more negative.

Observations were made concerning the selectivity of group 1a ions on the leak system. The leak conductance was measured in experiments where the test solution contained high concentration of the studied ion (see Methods). Some experiments with Li<sup>+</sup> were also included. The findings concerning the leak conductance were similar to those of Hille (1971) on *Rana pipiens*. The selectivity sequence found in the present experiments was  $K > Rb > Cs > Na > Li$ . This sequence for *Arenaria laevis* differs in one aspect from that for *Rana pipiens* where  $Cs > Rb$ .

Ba<sup>2+</sup> and La<sup>3+</sup> were found to decrease the leak conductance (Table 2). The effect was more pronounced in high [K<sup>+</sup>] solution than in low.

## DISCUSSION

In the present investigation a comparative analysis was made of the effects on the nodal membrane of the alkali metal ions Rb<sup>+</sup> and Cs<sup>+</sup> (group 1a periods 5 and 6), the alkaline earth metal ions Sr<sup>2+</sup> and Ba<sup>2+</sup> (group 2a period 5 and 6) and the transition metal ion La<sup>3+</sup> (group 3, period 6). The main find-

ing concerned effects on the potassium system: the ions of period 6 in the periodic system (Cs<sup>+</sup>, Ba<sup>2+</sup> and La<sup>3+</sup>). Similar effects but less pronounced were also obtained by the ions of period 5 (Rb<sup>+</sup> and Sr<sup>2+</sup>). The effects included (a) a reduction of steady state  $P_{K^+}$  at positive potential steps which increased with atomic number and (b) a reduction of  $I_{K^+}$  which decreased with atomic number.

Physico-chemical properties that change systematically with atomic number within period 6 in period 5 are for instance ionic radius, hydration energy, electronegativity, basic character and tendency for the metal ion to acquire covalent character when combined with anions (see for instance Duffy 1974). Values for some of these properties are summarized in Table 3. The complexing power of metal ions depends on their charge to radius ratio and in the case of transition metal ions on their ligand field stabilisation energy (cf. the Irving-Williams series of stability). At present it is impossible to explain the described effect on the potassium system by any single physico-chemical property of the types mentioned. It should here be pointed out that while Sr<sup>2+</sup> and Ba<sup>2+</sup> differed markedly in their effect on the steady state  $P_{K^+}$  at large potentials, they differed very little in effect on peak  $P_{K^+}$ .

Some findings in the present investigation are of special relevance for current hypotheses of the molecular mechanism of the potassium system. Barium decreased the steady state  $I_{K^+}$  calculated from the outward  $I_{K^+}$  at positive potential steps.

# Effects of physical training on metabolism of connective tissues in young mice

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SUOMINEN A, KIISKINEN A. & HEIKKINEN E. Effects of physical training on metabolism of connective tissues in young mice. *Acta Physiol Scand* 1980 108: 17-22. Received 13 March 1979. ISSN 0001-6777. Department of Public Health, University of Jyväskylä, Finland.

The effects of physical training on the metabolism of collagen, calcium and glycosaminoglycans in various connective tissues were studied in male NMRI mice. The mice (I) be trained and their controls were about 3 weeks old (expt. I) and 8 weeks old (expt. II) at the commencement of training. The training was performed on a 5° inclined treadmill 5 days a week for 4 weeks in expt. I and for 3 weeks in expt. II. The daily exercise time was progressively increased from 20 min in the first week up to 80 min in the third week. The incorporation of <sup>3</sup>H-proline to collagen hydroxyproline was increased by training in long bones, skeletal muscle and Achilles tendon, whereas the incorporation of <sup>35</sup>S-sulphate into glycosaminoglycans of bones was lower for the trained compared to the control mice. The differences between the two groups in the incorporation of <sup>45</sup>Ca were negligible. The results indicate that the metabolism of collagen is accelerated by physical training in several connective tissues in young rapidly growing mice, whereas the metabolism of glycosaminoglycans remains unaffected or is even retarded.

**Key words:** Metabolism collagen, calcium, glycosaminoglycans, connective tissues, physical training mice.

Physical training has been shown to influence both physical and chemical properties of connective tissues (see Booth & Gould 1975; Kiiskinen 1976). The effects of training on the metabolism of different connective tissue components have, however, been scarcely investigated. Anderson et al. (1971) found an indication of an accelerated mineral collagen turnover through Sr and <sup>3</sup>H-hydroxyproline excretion studies in pigs trained for 2 weeks. Enhanced collagen metabolism of long bones and Achilles tendon was also demonstrated by H<sup>3</sup> proline incorporation measurements in relatively old mice after one month training (Heikkinen & Mäenpää 1977). In addition, some earlier human studies support the idea of accelerated mineral metabolism evoked by physical exercise (Ehrenberg & Gordon 1961; Ragam & Briscoe 1964). The authors are not aware of any report describing the effects of training on the metabolism of ground substance glycosaminoglycans.

This study attempted to determine the effects of a few week endurance-type training on the

metabolism of collagen, calcium and glycosaminoglycans in various connective tissues of young male mice.

## MATERIAL AND METHODS

### Animals and experimental conditions

Two separate experiments were carried out. Male NMRI-mice (3 weeks old in expt. I and 8 weeks old in expt. II) were randomly assigned to test and control groups. The animals to be trained were gradually adapted to running on a treadmill. The training was performed on a 5° inclined motor-driven treadmill at a speed of 18 m/min, 5 days a week for 4 weeks in expt. I and for 3 weeks in expt. II. The daily exercise was performed in two sessions, the first in the morning and the second in the afternoon. The training time was progressively increased from 20 min in the first week up to 2-40 min in the third week. The animals were weighed both at the beginning and at the end of the training period. The heart weights served as an indication of training efficiency. The trained as well as the control animals were kept in normal laboratory conditions and pelleted mouse diet (Hankkylä, Finland) and tap water were given *ad libitum*.

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# Effects of physical training on metabolism of connective tissues in young mice

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SUOMINEN H., KIISKINEN A. & HEIKKINEN E. Effects of physical training on metabolism of connective tissues in young mice. *Acta Physiol Scand* 1980 108: 17-22. Received 13 March 1979. ISSN 0001-6772. Department of Public Health, University of Jyväskylä, Finland.

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**Key words:** Metabolism, collagen, calcium, glycosaminoglycans, connective tissues, physical training, mice.

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This study attempted to determine the effects of a few week endurance type training on the

metabolism of collagen, calcium and glycosaminoglycans in various connective tissues of young male mice.

## MATERIAL AND METHODS

### Animals and experimental conditions

Two separate experiments were carried out. Male NMRI-mice (3 week old in expt. I and 8 weeks old in expt. II) were randomly assigned to test and control groups. The animals to be trained were gradually adapted to running on a treadmill. The training was performed on a 5° inclined motor-driven treadmill at a speed of 18 m/min 5 days a week for 4 weeks in expt. I and for 3 weeks in expt. II. The daily exercise was performed in two sessions, the first in the morning and the second in the afternoon. The training time was progressively increased from 2-10 min in the first week up to 2-40 min in the third week. The animals were weighed both at the beginning and at the end of the training period. The body weight served as an indication of training efficiency. The trained as well as the control animals were kept in normal laboratory conditions and pelleted mouse diet (Hankkija, Finland) and tap water were given *ad libitum*.

Table 1 Body weights, tissue weights and hematocrite values in trained and control mice. Mean  $\pm$  S.D. and number of observations are given

Experiment	Variable	Trained mice	Control mice
I	Initial body weight g	17.4 $\pm$ 1.6 (4)	17.5 $\pm$ .3 (36)
	Final body weight g	32.4 $\pm$ 3.9 (28)	31.7 $\pm$ 3.9 (30)
	Dry weight of heart mg	34.1 $\pm$ 5.7 (28)	31.6 $\pm$ 4.9 (30)
	Dry weight of humerus mg	38.7 $\pm$ 5.0* (28)	34.8 $\pm$ 4.3 (30)
	Dry weight of femur mg	75.8 $\pm$ 12.1 (28)	67.9 $\pm$ 9.5 (30)
	Dry weight of m. rectus femoris, mg	83.3 $\pm$ 1.9* (28)	77.1 $\pm$ 1.1 (30)
	Dry weight of Achilles tendons mg	3.04 $\pm$ 0.38 (28)	.88 $\pm$ 0.43 (30)
	Hematocrite %	44.0 $\pm$ 3.7 (28)	44.3 $\pm$ 3.4 (30)
II	Initial body weight g	33.0 $\pm$ .8 (28)	33.0 $\pm$ 1.4 (36)
	Final body weight g	34.0 $\pm$ 2.6 (—)	36.7 $\pm$ 3.0 (—)
	Dry weight of heart mg	36.7 $\pm$ 5.2 (28)	34.7 $\pm$ 4.6 (22)
	Hematocrite %	41.8 $\pm$ 4.0 (—)	44.6 $\pm$ 3.0 (—)

$P < 0.10$   $P < 0.01$  when compared with control group

#### Metabolism of collagen and calcium (experiment I)

The metabolism of collagen and calcium was examined simultaneously by using a double labelling technique at the end of the training period.  $60 \mu\text{Ci}$   $^3\text{H}$ -proline (TRA 82 Radiochemical Centre, Amersham) and  $^{40} \mu\text{Ci}$   $^{45}\text{Ca}$  (CES-3 Radiochemical Centre, Amersham) were diluted in a physiological saline solution and injected i.p. into each mouse under ether anesthesia. The incorporation was allowed to continue for 4, 10, 4 and 96 h. After each incorporation period, 7–8 animals from the two groups were killed by decapitation under anesthesia and blood samples were taken in EDTA tubes for hematocrite (micro-method) and plasma radioactivity measurements. Plasma  $^3\text{H}$  and  $^{45}\text{Ca}$  radioactivities were measured by a liquid scintillation counter (LKB Wallac 81000) as described for the bone (see under). Humerus, femur, m. rectus femoris and Achilles tendons were removed and stored at  $-20^\circ\text{C}$  for subsequent analyses.

B. Humerus and femur were freed from muscles, soft connective tissues and bone marrow and analyzed separately. The bones were dried at  $+90^\circ\text{C}$  for two days, weighed (Mettler H20T) and hydrolyzed in 6 M HCl for 16

h at  $170^\circ\text{C}$ . The hydrolyzates were evaporated to dryness and dissolved in 5 ml of distilled water. Aliquots were taken for the measurement of both calcium concentration and  $^{45}\text{Ca}$ -radioactivity. Calcium concentration was analyzed by an atomic absorption spectrophotometer (Ultrascan SP90A) applying the method of Pyburn et al. (1970).  $^{45}\text{Ca}$ -activity was determined by the liquid scintillation counter by setting the discriminator to the energy level where no  $^3\text{H}$  activity was present. For the determination of  $^3\text{H}$  radioactivity, 0.5 ml of the solution was diluted into 1.5 ml of distilled water and calcium was precipitated with 0.5 ml of saturated ammonium oxalate at pH 5 (cf. Uitto & Laitinen 1968). An aliquot of the supernatant was used for the counting of total  $^3\text{H}$  radioactivity (Prockop & Ebert 1963). The rest of the dissolved hydrolyzate (4 ml) was analyzed for  $^3\text{H}$  hydroxyproline as described by Juss & Prockop (1966). In all radioactivity measurements a similar scintillant (15 g POP and 50 mg POPOP in 11 toluene, together with 0.6 l methylcellosolve) was used. The results were counted as dpm by using an external standard channel ratio method. The counting parameters were calculated using quenching series of  $^{45}\text{Ca}$  and  $^3\text{H}$ .

Table 2 Concentration of hydroxyproline, calcium and hexosamines ( $\mu\text{g/g}$  dry weight) in various tissues of trained and control mice. Mean  $\pm$  S.D. and number of observations are given

Experiment	Tissue	Variable	Trained mice	Control mice
I	Humerus	Hydroxyproline	18.3 $\pm$ 3.8 (17)	15.9 $\pm$ 3.4 (30)
		Calcium	226 $\pm$ 8 (27)	23 $\pm$ 8 (30)
	Femur	Hydroxyproline	17.0 $\pm$ 3.9 (36)	17.5 $\pm$ 3 (30)
		Calcium	5 $\pm$ 19 (38)	7 (—) (30)
	M. rectus femoris	Hydroxyproline	1.97 $\pm$ 0.7 (28)	0.1 $\pm$ 0.7 (30)
	Achilles tendon	Hydroxyproline	90.5 $\pm$ 9.8 (38)	91.1 $\pm$ 8.9 (30)
II	Humerus + femur	Hexosamines	1.34 $\pm$ 0.15 (—)	1.36 $\pm$ 0.16 (—)
		Hexosamines	53.0 $\pm$ 31 (1)	50.0 $\pm$ 1 (1)
	Achilles tendon	Hexosamines	1.80 $\pm$ 0.18 (—)	1.83 $\pm$ 0.17 (1)
	Skin	Hexosamines	—	—

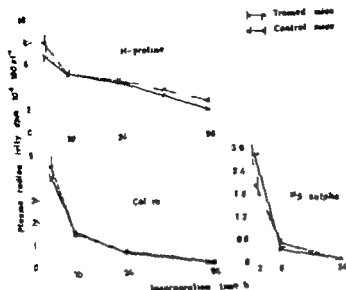


Fig. 1. Plasma radioactivity in trained and control mice. Points represent mean  $\pm$  S.E. of 3-8 observations.  $P < 0.10$ ,  $P < 0.05$  when compared with control group.

proline made in the laboratory immediately before the experiments.

**Mice and tissues.** The ulnae, radius femora and Achilles tendons of both legs are pooled and analyzed as described for the bones. Only the  $^3\text{H}$ -proline incorporation results will however be presented.

#### Measurement of glycoconjugates in *peroneus III*

The metabolism of glycoconjugates was studied by  $^{35}\text{S}$ -sulphate incorporation technique at the end of the training period. Isotope  $^{35}\text{S}$ -sulphate ( $^{35}\text{S}$ -P Radiochemical Centre, Amersham) as diluted in physiological saline solution and 2.5  $\mu\text{Ci/g}$  body weight was given *p.o.* under ether anaesthesia. After 2, 8 and 4 h incorporation times, the trained and control animals (2-8 mice in each group) were killed by decapitation under anaesthesia, and blood samples were taken for haematocrite and plasma radioactivity measurement. Humerus, femur, Achilles tendon and skull were removed and stored at  $20^\circ\text{C}$  for further analyses.

**Bones.** Humerus and femur of the right side were combined and blended together as one sample. After removing the muscles, soft connective tissues and bone marrow, the bones were decalcified in 5 ml of 0.05 M EDTA for 3 days. Each bone sample was crushed in mortar and homogenized 1 time for 5 s in 2 ml of ice-cold distilled water by using Sorvall Omac mixer (micro head) kept in an ice bath. The sample was dialyzed against cold running tap water on rough freeze-dried and weighed 10 times, hydrolyzed in 1 M HCl for 16 h at  $100^\circ\text{C}$ , and an aliquot was taken for the determination of total  $^{35}\text{S}$ -radioactivity. The rest of the hydrolyzate was used for the determination of the specific radioactivity of hexosamines. Hexosamines were freed from interfering heterogens by means of Dowex 50 cation exchange resin according to Bown (1953)

after which the concentration was determined as described by Oett & Bertram (1966). The  $^{35}\text{S}$ -sulphate radioactivities were measured by the liquid scintillation counter by using scintillant scintillator and decay calculation as in expt. I.

**Tendon.** The Achilles tendons of the animals were pooled and homogenized ten times for 3 s in 2 ml of ice-cold distilled water by using Potter-Elvehjem-type homogenizer kept in an ice bath. Further analyses were performed as for the bones.

**Skin.** The skin was shaved, and subcutaneous tissue was scraped off with blunt knife. A sample of dorsal skin was obtained with 20 mm punch cut into small pieces and homogenized and analyzed in the same way as the Achilles tendons.

#### Statistical method

The results were statistically evaluated by Student's *t*-test (2P) for non-correlating means.

## RESULTS

Table 1 presents the body weight, tissue weight and haematocrite values in the trained and control mice. The final body weight of the trained matured mice (expt. III) was significantly lower compared to that of the control mice of similar ages, whereas there were no differences in body weight values between the two younger groups (expt. I). The dry weight of both humerus and femur were significantly higher for the trained versus control mice in expt. I. The heart and skeletal muscle weights also tended to be higher in the trained growing mice. All of the

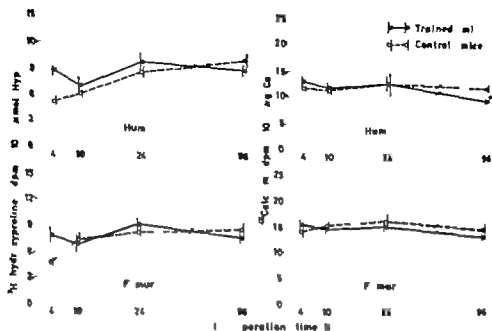


Fig. 2 Incorporation of  $^3\text{H}$  proline and  $^{45}\text{Ca}$  into connective tissue of bones in trained and control mice in expt 1. Points represent mean  $\pm$  SE of 4-8 observations.  $P < 0.05$   $P < 0.01$  when compared with control group.

hematocrite values were at the "normal" level although there was a significant difference between the two groups in expt 11.

As shown by Table 2 there were no significant differences between the trained and control mice in the concentrations of hydroxyproline, calcium or hexosamines in any of the studied tissues.

The plasma radioactivities after the various incorporation periods appear in Fig. 1. Except for some variation in the  $^{35}\text{S}$ -sulphate activity the curves were similar for trained and untrained animals.

The results of the incorporation of  $^3\text{H}$  proline,  $^{45}\text{Ca}$  and  $^{35}\text{S}$ -sulphate into various connective tissues are shown in Figs 2-4. In every tissue studied the formation of  $^3\text{H}$  hydroxyproline was significantly higher for the trained compared to the control mice after 4 h of incorporation. An opposite difference between the two groups was observed in the incorporation of  $^{35}\text{S}$ -sulphate into bones. Although the incorporation of  $^{45}\text{Ca}$  was very similar to that of  $^3\text{H}$  proline, the differences between the trained and control mice were negligible.

## DISCUSSION

The enhanced weight development of long bones in the trained growing mice (expt 1) is in agreement

with the earlier findings of Kliskinen (1977) as well as Saville & White (1969). The advanced growth of certain tissues did not, however, appear as differences between the two groups in the gain of body weight. As in expt 11 (Table 1) training rather retards the increase of total body weight with aging, presumably by preventing the accumulation of fat. The results further indicate that the hypertrophy of heart and skeletal muscle is more pronounced during the period of rapid growth.

The concentrations of hydroxyproline, calcium and hexosamines in the tissues studied were not changed by the exercise program used in the present experiments. Neither did Tipton et al. (1970) observe any changes in the hexosamine concentration of various knee ligaments in trained dogs. Some recent findings (Kliskinen & Heikkinen 1978) however suggest that animal age and the duration of training may have an influence on the response of long bones to exercise.

The enhanced incorporation of radioactive proline to collagen hydroxyproline in bones, skeletal muscle and Achille tendon of the trained mice particularly during the first hours of the incorporation period suggests that the metabolism of collagen is accelerated by training. This effect is probably not limited to the rapid growth period only because a similar observation was also made on rela-

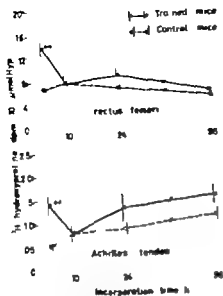


Fig. 3 Incorporation of  $^3\text{H}$ -proline into connective tissue of skeletal muscle and tendon in trained and control mice in expt. 1. Points represent mean  $\pm$  S.E. of 3-8 observations.  $P < 0.01$  when compared with control group.

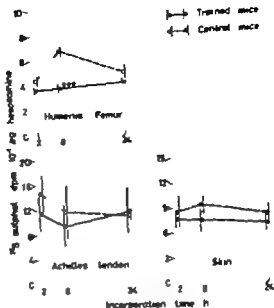


Fig. 4 Incorporation of  $^{35}\text{S}$ -sulphate into connective tissue of bones, tendon and skin in trained and control mice in expt. 2. Points represent mean  $\pm$  S.E. of 5-11 observations for bones and skin and that of 3-4 observations for Achilles tendon.  $^{***}P < 0.001$  when compared with control group.

tively old mice (Heikkinen & Vuori 1972). Further evidence of a training-induced acceleration of collagen metabolism is given by the increased activity of prolyl hydroxylase in rat skeletal muscle after compensatory hypertrophy (Turto *et al.* 1974) in mice after treadmill training (Suominen & Heikkinen 1975) and in humans after endurance-type training (Suominen & Heikkinen 1975b; Suominen *et al.* 1977).

The interpretation of the present results on collagen metabolism is disadvantaged by the lack of clear decline in radioactivity during the follow up period and by a somewhat odd 'fall' in radioactivity in the trained group after 10 h of incorporation (see Fig. 2.3) which meant that the actual turnover times could not be deduced. The same difficulties are also true in the estimation of calcium metabolism. In addition, several different pools of calcium exist in long bones (e.g. Laitinen 1976) which makes it difficult to indicate what fraction of bone calcium could possibly be influenced by exercise.

The incorporation of  $^{35}\text{S}$ -sulphate particularly into long bones, was somewhat unexpectedly decreased by training suggesting that the response to physical stress is not similar in the different components of connective tissues. Because this ob-

servation is of great interest in respect of the age related changes of connective tissues, additional information is needed before definite conclusions can be drawn.

The observed changes in the metabolism of connective tissues evoked by physical training were not quantitatively very dramatic, but it has to be remembered that even small consistent changes may be biologically important in tissues which undergo systematic and progressive life-long alterations (cf. Balazs 1977).

This study was supported by grants from the Finnish Research Council for Physical Education and Sport (Ministry of Education) and the Academy of Finland. The authors also wish to thank Mrs. Rajja Niskanen and Mr. Erkki Heikkilä for their skilful technical assistance.

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# The effect of divalent and trivalent cations on the sodium permeability of myelinated nerve fibres of *Xenopus laevis*

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BRISVÄR, T. The effect of divalent and trivalent cations on the sodium permeability of myelinated nerve fibres of *Xenopus laevis*. *Acta Physiol Scand* 1980; 108: 23-29. Received 30 March 1979. ISSN 0001-6772. Department of Clinical Neurophysiology, Huddinge Sjukhus, Huddinge, and Nobel Institute for Neurophysiology, Karolinska Institute, Stockholm, Sweden.

The effect of external  $\text{Ca}$ ,  $\text{Mg}$ ,  $\text{Sr}$ ,  $\text{Ba}$  and  $\text{La}$  concentration was studied in potential clamp experiments on single myelinated nerve fibres of the frog (*Xenopus laevis*). A increase in cationic concentration caused positive shift of the  $\text{Na}$  permeability ( $P_{\text{Na}}$ ) curve along the potential axis, decrease in  $\text{max } P_{\text{Na}}$  and decrease in the slope of the  $P_{\text{Na}}$  curve. The effectiveness of  $\text{Mg}$ ,  $\text{Sr}$  and  $\text{Ba}$  was similar and about 0.6 compared to that of  $\text{Ca}$ , whereas  $\text{La}$  is about 80 times more effective than  $\text{Ca}$ .

Key words:  $\text{Ca}$ , surface charges, potential clamp, myelinated nerve

The concentration of  $\text{Ca}$  in the external solution affect the excitability of nerve and muscle. Frankenhaeuser & Hodgkin (1957) found that an e-fold increase in  $[\text{Ca}]$  shifted the curve relating peak  $\text{Na}$  conductance to membrane potential about 9 mV along the potential axis in a direction such that larger positive potential steps now were required to bring about an increase of the  $\text{Na}$  conductance by given amount. This result was based on a potential clamp investigation on squid axons and the  $\text{Ca}$ -concentrations used were in the range of 4.4 to 11 mM. Frankenhaeuser (1957) estimated on the myelinated nerve fibre the change in the threshold potential for spike initiation to about 6 mV per e-fold change in  $[\text{Ca}]$ . Hille (1968) described a somewhat larger shift, 8.7 mV, of the  $\text{Na}$  permeability in the frog node associated with the same change in  $[\text{Ca}]$ .

Other cations than  $\text{Ca}^{2+}$  influence the relation between  $\text{Na}$  conductance (or permeability  $P_{\text{Na}}$ ) and membrane potential. In squid axons changes in  $[\text{Mg}]$  are associated with similar shifts of the sodium conductance curve along the potential axis, but the effectiveness of  $\text{Mg}$  is about 0.6 of that of  $\text{Ca}$  (Frankenhaeuser & Hodgkin 1957). In the myelin-

ated nerve fibre excitability measurements indicated a somewhat smaller value, 0.4 (Frankenhaeuser & Meves 1958). Similar measurements were made on lobster nerve by Blaustein & Goldman (1968). According to these measurements the effectiveness relative to that of  $\text{Ca}$  would be 0.2 of  $\text{Mg}$  but larger than 1.0 of  $\text{Ba}$ . The trivalent cation  $\text{La}$  has been shown to affect the  $\text{Na}$  conductance of the lobster giant fibre in a  $\text{Ca}$ -like fashion but with a 20 times greater potency (Takata et al 1966). In crayfish axons D'Arrigo (1973) found that the effectiveness of  $\text{Ca}$ ,  $\text{Mg}$ ,  $\text{Sr}$  and  $\text{Ba}$  was essentially equal upon threshold membrane potential for spike initiation while it was slightly greater for  $\text{Co}$  and  $\text{Ni}$  and much greater (about 60 times) for trivalent cations. On frog fibres Hille, Woodhull & Shapiro (1975) showed that the effectiveness of divalent cations on the position of the  $P_{\text{Na}}$  curve followed the order  $\text{Ba} \approx \text{Sr} < \text{Mg} < \text{Ca} \approx \text{Mn} < \text{Ni} < \text{Zn}$  an effectiveness which increased for all ions at higher pH. Vogel (1974) compared the effect of  $\text{Ca}$  and  $\text{La}$  on the permeability parameters in the myelinated nerve fibre of the frog. He found the effectiveness

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The effect of external  $\text{Ca}$ ,  $\text{Mg}$ ,  $\text{Sr}$ ,  $\text{Ba}$  and  $\text{La}$  concentrations, as studied in potential clamp experiments on single myelinated nerve fibres of the frog (*Xenopus laevis*). An increase in calcium concentration caused positive shift of the  $\text{Na}$  permeability ( $P_{\text{Na}}$ ) curve along the potential axis, decrease in its  $P_{\text{Na}}$  and decrease in the slope of the  $P_{\text{Na}}$  curve. The effectiveness of  $\text{Mg}$ ,  $\text{Sr}$  and  $\text{Ba}$  was similar and about 0.6 compared to that of  $\text{Ca}$ , whereas  $\text{La}$  was about 80 times more effective than  $\text{Ca}$ .

**Key words:**  $\text{Ca}$ , surface charges, potential clamp, myelinated nerve.

The concentration of  $\text{Ca}$  in the external solution affects the excitability of nerve and muscle. Frankenhaeuser & Hodgkin (1957) found that an e-fold increase in  $[\text{Ca}]$  shifted the curve relating peak  $\text{Na}$  conductance to membrane potential about 9 mV along the potential axis in a direction such that larger positive potential steps now were required to bring about an increase of the  $\text{Na}$  conductance by given amount. This result was based on a potential clamp investigation on squid axons and the  $\text{Ca}$ -concentrations used were in the range of 4.4 to 112 mM. Frankenhaeuser (1957) estimated on the myelinated nerve fibre the change in the threshold potential for spike initiation to about 11 mV per e-fold change in  $[\text{Ca}]$ . Hille (1968) described a somewhat larger shift, 2.7 mV, of the  $\text{Na}$  permeability in the frog node associated with the same change in  $[\text{Ca}]$ .

Other cations than  $\text{Ca}^{2+}$  influence the relation between  $\text{Na}$  conductance (or permeability  $P_{\text{Na}}$ ) and membrane potential. In squid axons changes in  $[\text{Mg}]$  are associated with similar shifts of the sodium conductance curve along the potential axis, but the effectiveness of  $\text{Mg}$  is about 0.6 of that of  $\text{Ca}$  (Frankenhaeuser & Hodgkin 1957). In the myelinated

nerve fibre excitability measurements indicated a somewhat smaller value, 0.4 (Frankenhaeuser & Meves 1958). Similar measurements were made on lobster nerve by Blaustein & Goldman (1968). According to these measurements the effectiveness relative to that of  $\text{Ca}$  would be 0.4 of  $\text{Mg}$  but larger than 1.0 of  $\text{Ba}$ . The trivalent cation  $\text{La}$  has been shown to affect the  $\text{Na}$  conductance of the lobster giant fibre in a  $\text{Ca}$ -like fashion, but with a 20 times greater potency (Takata et al. 1966). In crayfish axons D'Arrigo (1973) found that the effectiveness of  $\text{Ca}$ ,  $\text{Mg}$ ,  $\text{Sr}$  and  $\text{Ba}$  was essentially equal upon threshold membrane potential for spike initiation, while it was slightly greater for  $\text{Co}$  and  $\text{Ni}$  and much greater (about 60 times) for trivalent cations. On frog fibres Hille, Woodhull & Shapiro (1975) showed that the effectiveness of divalent cations on the position of the  $P_{\text{Na}}$  curve followed the order  $\text{Ba} \sim \text{Sr} < \text{Mg} < \text{Ca} \sim \text{Mn} < \text{Ni} < \text{Zn}$ , an effectiveness which increased for all ions at higher pH. Vogel (1974) compared the effect of  $\text{Ca}$  and  $\text{La}$  on the permeability parameters in the myelinated nerve fibre of the frog. He found the effectiveness

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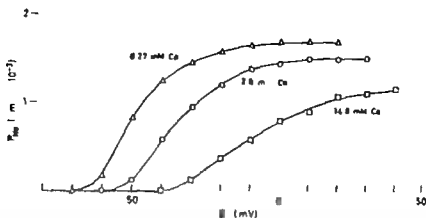


FIG. 1. Effect of  $Ca^{2+}$  concentration on the relation between the peak sodium permeability and membrane potential change  $P_{Na}$  calculated from measurements of peak value of Na current in positive potential steps to various amplitudes indicated on abscissa. Membrane potential  $U = -130$  mV between test pulses. Temperature  $5^{\circ}C$ . Fibre I.

of La to be 55 times that of Ca calculated from the shift of the  $P_{Na}$  curve along the potential axis associated with changes in the concentration of these ions.

The concentration of univalent cations affects the position of the  $P_{Na}$  curve on the potential axis like the concentration of divalent and polyvalent cations (Brismar 1973; Hille et al. 1975).

It has been suggested that  $Ca^{2+}$  acts on the excitability of nerve through the electrical screening of negative charges, which are assumed to be fixed on the external surface of the membrane (McLaughlin, Szabo & Eisenman 1971; Brismar 1973; D'Arigo 1973; Vogel 1974; Hille et al. 1975). Ca would thereby dependent on its concentration change the electric field in the membrane. The hypothesis would in the absence of further complications require that cations of the same valence have essentially the same effectiveness.

The present investigation was designed to test this hypothesis. Measurements were made of the dependence of the  $P_{Na}$  vs. potential curve on the concentration of the divalent cations Ca, Mg, Sr and Ba. Similar measurements were further made with solutions containing the trivalent cation La. All these ions caused a qualitatively similar displacement of the  $P_{Na}$  curve along the potential axis. Ca had a larger effect than the other divalent ions while La was much more active than Ca.

## METHODS

Single myelinated nerve fibres were isolated from the sciatic nerve of the frog (*Xenopus laevis*). The potential clamp technique developed by Dodge & Frankenhaeuser

(1958) was used with some minor modifications (cf. Brismar & Frankenhaeuser 1977).

Measurements were made of how the peak nodal currents were associated to potential steps of various amplitudes. The peak  $P_{Na}$  was calculated from these measurements according to Dodge & Frankenhaeuser (1959) using the constant field equation, i.e. the expression for the single ionic current which can be calculated from the Nernst-Planck equation if the electric field inside the membrane is constant (Goldman 1943; Hodgkin & Katz 1949). Such permeability measurements were carried out with the fibre in a number of different concentrations of divalent and polyvalent cations.

**Solutions.** The solutions contained 11.0 mM NaCl, 5 mM KCl, 4 mM Tris buffer (Sigma, pH 7.8 at  $5^{\circ}C$ ) and different concentrations (0, 7.0 or 14.8 mM) of  $CaCl_2$  (or  $MgCl_2$ ,  $BaCl_2$ ,  $SrCl_2$ ) or one of the following ( $LaCl_3$ —91 or 670 nM, 5.37 or 770  $\mu M$ ). As a reference solution the solution containing 11 mM  $CaCl_2$  was used.

**Voltage-clamp.** Membrane potential ( $U$ ) is given as inside potential minus outside potential.

## RESULTS

Potential clamp experiments were performed on single myelinated nerve fibres of the frog (*Xenopus laevis*). Measurements were made of the effect of test solutions with different concentrations of divalent and trivalent cations on the Na permeability properties of the nodal membrane.

The action of  $[Ca]$  on the relation between the peak  $P_{Na}$  (calculated from the constant field equation) and the membrane potential ( $U$ ) is shown in Fig. 1. In agreement with the findings in several earlier investigations (see introduction) changes in the  $[Ca]$  affected the position of the  $P_{Na}$  curve on the potential axis. However, the effect was more complex than merely a parallel shift of the permea-

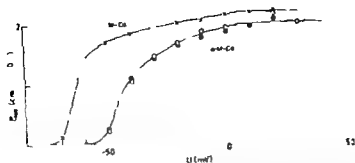


Fig. 2. Effect of zero-Ca solution on peak sodium permeability  $P_{Na}$  calculated from measurements of peak value of initial current at positive potential steps to various amplitudes given on abscissa. Membrane potential  $U = 130$  mV between test pulses. Solutions applied in the order 2.0 mM-Ca (O), zero Ca ( ) and 11 mM-Ca (●). Temperature 37°C. Fibre 1.

bility curve along the potential axis. The maximum peak  $P_{Na}$  at large positive potential steps was smaller in high  $[Ca]$  as compared to low. Furthermore the steepness of the  $P_{Na}$  curve decreased with an increase in  $[Ca]$ . These effects were reversible. The effect of the  $[Ca]$  on the slope of the  $P_{Na}$  curve was a regular finding. The slope was steeper in low  $[Ca]$  also when the  $P_{Na}$  curves were scaled in units of the max. peak  $P_{Na}$ .

Fig. 2 illustrates an experiment with the node exposed to a solution without divalent cations containing only 112 mM-NaCl, 2.5 mM-KCl and 2.5 mM-Tris buffer. The peak  $P_{Na}$  curve was shifted in negative direction and the slope of the curve increased. The limiting value of the peak  $P_{Na}$  at large positive potential steps was somewhat larger. The action of a Ca-free solution was not qualitatively different from that of a low  $[Ca]$  solution shown in Fig. 1.

Experiments were further carried out with solutions containing various concentrations of one of the following cations:  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Sr^{2+}$  and  $Ba^{2+}$ . Nerve axons run down more rapidly in low  $[Ca]$  solutions than in high (Frankenhaeuser & Hodgkin 1957). Experiments in low  $[Ca]$  and in solutions containing other divalent cations than Ca were therefore performed rapidly. Frequent control runs were made in reference solution containing 2.0 mM-Ca in order to give a check on possible irreversible changes in the membrane currents following the exposure to test solutions. Clamp runs showing irreversible changes such as a decreased ratio between specific permeability and leak conductance were discarded. The calculations of ef-

fects associated with a test solution were made from comparisons with the mean of the peak  $P_{Na}$  values in the reference solution preceding and following the test solution. Errors introduced by small continuous changes in the permeability properties were minimized by this procedure.

Collected data from measurements performed with various concentrations of Ca, Mg, Sr, Ba and La are given in Fig. 3, 4 and 5. Similar to the action of high  $[Ca]$  an increase in  $[Mg]$ ,  $[Sr]$  and  $[Ba]$  was associated with: (i) a shift of the  $P_{Na}$  curve in positive direction along the potential axis; (ii) a decrease in the maximum peak  $P_{Na}$  at large positive potential steps; and (iii) a decrease in the steepness of the peak  $P_{Na}$  curve. The action of Mg, Sr and Ba was thus very similar to that of Ca. Fig. 3 represents a plot of the  $P_{Na}$  shift (mV) versus concentration (logarithmic scale) of tested cation. The measurements indicated that Ca was more effective than Mg, Sr and Ba. No quantitative difference was observed between Mg, Sr and Ba. The effectiveness of Mg, Sr and Ba relative to that of Ca was estimated to about 0.6. Changes in the cationic concentration caused larger permeability shifts at the high concentration range about 13 mV per e-fold concentration change at 15 mM and less than 7 mV at 0.27 mM. This is described by the smooth curves which in negative direction asymptotically approach the limiting value estimated in solutions without divalent or trivalent cations. The curves are calculated from the Guy-Chapman equation relating surface potential to ionic concentration at a certain density of fixed charges at the membrane surface (Grahame 1947 eqn. given in legend of Fig.

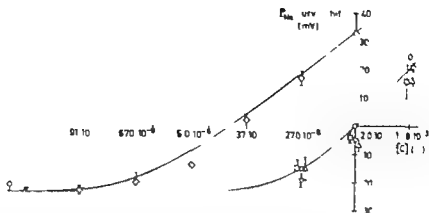


Fig. 3 Shift of  $P_{Na}$  curve vs. concentration of different polyvalent cation ( $C$  logarithmic scale). The shift (mV) was calculated from the membrane potential at which  $P_{Na}$  equals half its maximum in the test solution minus the potential similarly defined in the reference solution containing  $1.0 \text{ mM-Ca}$ .  $P$  calculated from the peak value of the Na current. The symbols refer to solutions containing either Ca (O), Mg (□), Sr (V), Ba (Δ) or La (◇). Mean values  $\pm$  S.D. Curves are solutions of  $\sigma^2 = k_e RT \sum c_i [\exp(-\phi_{Na} F/RT) - 1]$  where  $\sigma$  = charge density ( $C \text{ m}^{-2}$ ),  $\phi_{Na}$  = surface potential minus potential of the bulk solution (V),  $k_e$  = dielectric constant of water ( $= 81.90$  at  $5^\circ\text{C}$ ),  $\epsilon_0$  = permittivity of free space ( $= 8.85 \times 10^{-12} \text{ F m}^{-1}$ ),  $c_i$  = ionic concentration of solution ( $\text{M m}^{-3}$ ),  $v_i$  = valence of the ionic species,  $F$  = Faraday constant ( $= 96485 \text{ C}$ ),  $R$  = gas constant ( $= 8.31 \text{ J M}^{-1} \text{ K}^{-1}$ ),  $T$  = absolute temperature (K). This equation is a modification of equation (40) in Grahame (1947). The charge density  $\sigma$  was unknown and assumed to be  $-13 \times 10^{-4} \text{ C m}^{-2}$  which gave a good fit to the measured shift of the  $P_{Na}$  curve.

3) Earlier measurements of the effect of  $[Ca]$  on the  $P_h$  curve were satisfactorily fitted by this equation at a charge density of  $-5.5 \times 10^{-4} \text{ C m}^{-2}$  (Brismar 1973). The earlier data (Fig. 5 in Brismar 1973) indicated that  $[Ca]$  affected the  $P_{Na}$  curve more than the  $P_h$  curve. The present measurements of the  $P_{Na}$  curve shift was also better described by the equation at a higher assumed charge density  $-33 \times 10^{-4} \text{ C m}^{-2}$  (Fig. 3).

The concentration of the divalent cations further affected the size of the peak  $P_{Na}$  at large positive potential steps. The changes in max. peak  $P_{Na}$  were related to the shift in the  $P_{Na}$  curve (Fig. 4). The max. peak  $P$  is expressed in units of max. peak  $I_{Na}$  in the reference solution containing  $1.0 \text{ mM-Ca}$ . An increase in the divalent cationic concentration was clearly associated with a decrease in max. peak  $P_{Na}$ . With regard to this action the effectiveness of the various divalent cations was essentially the same. Like the permeability shift described in Fig. 3, the change in max. peak  $P$  was larger in the range of high cationic concentration.

Also with regard to the change in the slope of the  $P_{Na}$  curve the action of different divalent cations was very similar (Fig. 5). The  $P_{Na}$  curve was more steep in low cationic concentration; the slope of the

$P_{Na}$  curve as defined in the legend of Fig. 5 was approximately linearly related to the position of the  $P_{Na}$  curve on the potential axis (or the  $P_{Na}$  curve shift). In solutions without divalent cations  $P_{Na}$  was turned on within a very narrow potential range (Fig. 7).

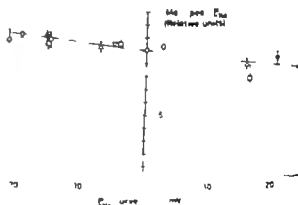


Fig. 4  $P_{Na}$  as function of  $P_{Na}$  curve shift in different cationic concentrations.  $P_{Na}$  = max. peak  $P_{Na}$  given as unit of its value in reference solution containing  $1.0 \text{ mM-Ca}$ . Max. peak  $P_{Na}$  calculated by the constant field equation from the peak value of the Na current at a voltage with large positive pulses. Membrane polarized to  $-170$  or  $-140 \text{ mV}$  between test pulses. Straight line drawn through reference point and fitted to the symbols.

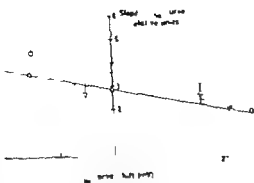


Fig. 6. Relation between slope and shift of  $P_{Na}$  curve measured at the point of the curve where  $P_{Na}$  was at maximum and expressed as arbitrary units defined as fractional change in  $P_{Na}$  (of max.  $P_{Na}$ ) per mV. Straight line fitted to the symbols. The symbols refer to solutions containing either Ca (O), Mg (□), Sr (▽) or Ba (Δ).

The action of the trivalent cation La was investigated. Similar to the divalent cations La acted upon the position of the peak  $P_{Na}$  curve on the potential axis (Fig. 6) the max. peak  $P_{Na}$  at large positive potential steps, and the slope of the  $P_{Na}$  curve. Distinct from the divalent cations La changed the Na permeability properties of the nodal membrane to some extent irreversibly. The decrease in the maximum peak  $P_{Na}$  as well as the shift of the permeability curve on the potential axis associated to 0.77 and 2.0 mM-La solutions were not immediately reversible in the reference solution following the exposure to La. The time course of the changes in the permeability properties are plotted in Fig. 7.

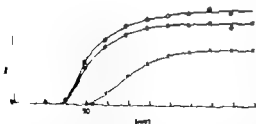


Fig. 7. Effect of La on N permeability. Peak  $P_{Na}$  calculated from measurements of peak value of N current associated with changes in membrane potential at different levels as indicated on abscissa. Membrane polarized to 120 mV between test pulses. Measurements in 0.77 mM-La (O) 1 min after beginning of solution change to 0.77 mM-La (□), and 1 min after beginning of washout in 0 mM-La (●). Temperature 15°C. Fibre No. 11.

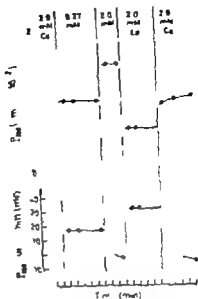


Fig. 8. Time course of La action on max. peak  $P_{Na}$  (top) and  $P_{Na}$  curve shift (bottom). Vertical lines mark beginning of solution change which required about 15 s. Each permeability curve as obtained from family of test pulses applied during about 30 s indicated on the abscissa in the time as the completion of each pulse family. Same action as in Fig. 6. Temp. 15°C.

The onset of La action was rapid and complete before the test pulses were applied. i.e. it cannot have required more than 30 s. During washout the permeability curve gradually shifted to the original position on the potential axis while the original amplitude of  $P_{Na}$  was incompletely restored. There was obviously a damage to the permeability mechanism associated with the exposure of the fibre to high [La] solutions. This effect may be separate from the reversible action of La on the potential dependence of the  $P_{Na}$  curve. Estimates of changes in max. peak  $P_{Na}$  in different [La] were therefore ambiguous. The quantitative description in Fig. 3 of the effect of various [La] was made from a comparison with the mean of the peak  $P_{Na}$  values in the reference solution preceding and following the test solution. The Figure shows, that a change from 2.0 mM-CaCl<sub>2</sub> to 1/80 as much LaCl<sub>3</sub> in the external solution would produce no permeability shift. The shift associated with an e-fold change in [La] was about 8 mV in the range 5  $\mu$ M to 2.0 mM. Below 5  $\mu$ M the effect of a decrease in [La] was small and approached the limiting value measured in solutions without divalent and trivalent cations.



## DISCUSSION

The effects of divalent and trivalent cationic concentration on the Na permeability properties of the nodal membrane have been described. From measurements of peak Na current at various membrane potentials the peak  $P_{Na}$  was calculated from the constant field equation (Dodge & Frankenhaeuser 1959) and plotted against the membrane potential. The analysis of such curves revealed that an increase in concentration of all cations investigated (Ca, Mg, Sr, Ba and La) shifted the  $P_N$  curve to more positive potentials and decreased the maximum  $P_{Na}$ . There was further a decrease in the slope of the  $P_{Na}$  curve in high concentrations of these ions. The effect on the permeability properties of an eightfold change in [Ca] was greater in the range of high [Ca]. This was also the case for changes in [Mg], [Sr], [Ba] and [La]. It is further known (see introduction) that the effect of [Ca] depends on the concentration of other salts present in the solution. For these reasons it is difficult to interpret the quantitative difference in Ca-effects on preparations with large difference in the salt concentration of the external solution. The obtained results were in agreement with investigations made by Frankenhaeuser (1957), Frankenhaeuser & Meves (1958), Hille (1968) and Vogel (1974) of different [Ca] in ordinary Ringer's solution on the nodal membrane of frog fibres. It is interesting to note that the Na permeability shift along the potential axis measured in Ringer's solution containing about 117 mM NaCl was about 1.5 times greater than the corresponding shift in solutions of 25.0 mM NaCl plus 90.0 mM KCl (Brismar 1973).

The relative effectiveness of other divalent cations compared to Ca was not dependent upon the concentration range. It was for all of them (Mg, Sr and Ba) estimated to about 0.6. A similar estimate was made by Frankenhaeuser & Hodgkin (1957) on the squid giant axon and by Frankenhaeuser & Meves (1958) on the frog myelinated nerve fibre. Hille et al. (1975) found the same difference between the effectiveness of Mg, Sr and Ba compared to Ca in frog fibres, but distinguished that Mg was slightly more effective than Ba and Sr. The lanthanum ion  $La^{3+}$  which was the only trivalent cation tested, had essentially the same action as the divalent cations, but its effectiveness was considerably greater. La differed from the divalent cations in the respect that solutions containing 0.7 mM La or more had partially irreversible effects

on the Na permeability properties. It has also been reported (Takata et al. 1966) that the effect of La on the Na permeability properties of lobster axon gradually increases during several minutes exposure. This was not the case for La-effect on the nodal membrane.

The greater effectiveness of Ca compared to other divalent cations belonging to the same group of elements has some definite implications on the hypothesis of Ca-action on the excitable membrane (see introduction). If it is assumed that the Ca affects the position of the Na permeability curve on the potential axis because of an interaction between Ca and fixed negative charges at the external membrane surface, it is necessary to make some assumptions which account for the difference between cations of the same valence. Several investigators (Gilbert & Ehrenstein 1969, Mody & Naumov 1970) have suggested that Ca could be to negatively charged groups on the surface and thereby neutralize the negative charge. The binding coefficient might differ between for example Na and Ca. In an investigation of the action of different divalent cations on the threshold for excitation of crayfish axons D'Arrigo (1974) found that pH influences the effectiveness of the different ions. This effect he explained from the action of pH on the electric field strength of fixed negative charges in the theory of alkaline-earth equilibrium selectivity (references in D'Arrigo 1974).

The basic idea in these hypotheses is that positive ions in the external solution interact with negatively charged groups on the external membrane surface. According to this, ions with higher valence would have considerably greater effect. So far the finding that La is much more effective than the divalent cations agrees with these hypotheses. This difference between the effectiveness of La compared to the divalent cations was approximately described by the model of cationic screening of fixed negative charges (density  $-33 \times 10^{-4}$  C/m<sup>2</sup>) at the nodal membrane surface. This value is considerably higher than earlier calculated to describe the effect of [Ca] on the  $P$  properties ( $-9.5 \times 10^{-4}$  C/m<sup>2</sup>, Brismar 1973). A similar difference between the effect of Ca and La on the  $I_{Na}$  and the  $I$  properties led Vogel (1974) to assume that the charge density was about  $-23 \times 10^{-4}$  C/m<sup>2</sup> at the Na permeability site and about  $-8.0 \times 10^{-4}$  C/m<sup>2</sup> at the K site.

Other effects of changes in divalent and trivalent cationic concentration such as the change in mu-

to  $P_{Na}$  and in the slope of the  $P_{Na}$  curve were recently not explained by the model. If however the effect of a negative surface potential is accounted for in the constant field equation as was done by Frankenhaeuser (1960) in his comparison of  $P_{Na}$  in frog and squid axons, the surface charge model of Ca action will also cover (at least qualitatively) the effect on max. peak  $P_{Na}$ . This model predicts changes in  $I_{Na}$  rectification which may be tested by the analysis of  $I_{Na}$  tails at repolarisation after positive pulses. Such an analysis was not included in the present investigation.

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## SUMMARY

Electrical clamp experiments were performed on single myelinated nerve fibres from the frog (*Xenopus laevis*). The  $Na$  permeability ( $P_{Na}$ ) properties of the nodal membrane were studied in different outside concentrations of the cations  $Ca$ ,  $Mg$ ,  $Sr$ ,  $Ba$  (divalent) and  $La$  (trivalent). An increase in cationic concentration (1) the  $P_{Na}$  curve was shifted in positive direction along the potential axis, (2) in  $Na$  ions  $P_{Na}$  decreased, and (3a) the slope of the  $P_{Na}$  curve decreased. The effectiveness of  $Mg$ ,  $Sr$  and  $Ba$  was similar about 0.6 compared to that of  $Ca$ , whereas  $La$  was about 80 times more effective than  $Ca$ . The hypothesis of slower action through screening of fixed negative charges on the nodal extracellular surface produced approximately the difference between  $La$  and  $Ca$  at an assumed charge density of  $33 \cdot 10^{-4} C m^{-2}$ .

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# Alkali metal cation transport through the human erythrocyte membrane by the anion exchange mechanism

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This work deals with the possibility that cations may be transported through the human red blood cell membrane as negatively charged ion pairs of the type XCO<sup>-</sup>. Passive sodium influx into human erythrocytes was increased 4-fold when 150 mM chloride was substituted with bicarbonate in the medium. This increased permeability to sodium was completely abolished when the cells were pretreated with specific inhibitor of anion transport (DIDS). Both bicarbonate-induced sodium influx and chloride exchange were linearly related to DIDS-binding. Both transport processes were completely inhibited by the binding of  $1 \times 10^6$  DIDS molecules per cell. The presence of bicarbonate and/or pretreatment of the cells with DIDS did not change the permeability of the erythrocyte membrane to K<sup>+</sup>, Rb<sup>+</sup> and Cs<sup>+</sup> to any significant extent. The experimental findings support the hypothesis that Li<sup>+</sup> and Na<sup>+</sup> but not K<sup>+</sup>, Rb<sup>+</sup> and Cs<sup>+</sup> form monovalent negatively charged ion pairs with CO<sup>-</sup> which traverse the membrane through the anion exchange system.

**Key words:** Anion transport, anionic cation transport, bicarbonate-carbonate ion pairs, cation transport, erythrocytes.

Sodium and lithium transport through the red cell membrane is very sensitive to the presence of HCO<sub>3</sub><sup>-</sup> which increases the membrane permeability to the cations. It has recently been demonstrated that the bicarbonate-stimulated Li<sup>+</sup> influx is abolished by inhibition of the specific anion exchange system of the red cell membrane (Funder, Tosteson & Wieth 1978). This finding gave support to the notion that the increase of Li<sup>+</sup> flux is due to the formation of negatively charged anion pairs (LiCO<sup>-</sup>) which can be transported by the anion pathway (Wieth 1970). The purpose of the present article is to investigate whether the bicarbonate-stimulated Na<sup>+</sup> transport also can be quantitatively related to the function of the anion exchange mechanism. In addition it is examined whether the stimulation of alkali metal cation transport with bicarbonate is limited to Li<sup>+</sup> and Na<sup>+</sup>. Potassium, rubidium, and cesium ions are not supposed to form ion pairs with carbonate. The ion pair hypothesis

therefore implies that bicarbonate will not stimulate passive transport of other alkali metal cations than Na<sup>+</sup> and Li<sup>+</sup>.

## METHODS

Freshly drawn heparinized human blood was centrifuged and the plasma and buffy coat were discarded. Unless otherwise noted the cells were washed 3 times in the medium in which the experiment was performed. After the final wash the cells were suspended at hematocrit of 30% and the cell suspension was incubated at 38°C and pH 7.4. Oxaluen 10 mM was added 5 min before start. The technique of incubation, control of pH and P<sub>CO<sub>2</sub></sub> and isolation of cells and media at 0°C has been described by Funder & Wieth (1967). Sodium and potassium were determined by flame photometry and the cell volume was determined by drying the cells to constant weight at 105°C for 24 h. All cellular concentrations were corrected for the trapping of 2.4 (ml/l) of extracellular medium in the packed cell column.

Pretreatment of erythrocytes with DIDS and the experimental procedure in the experiments with graded



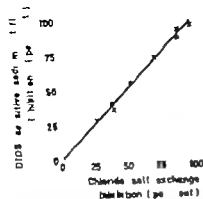


Fig. 1 Comparison of the DIDS-induced inhibition of chloride self-exchange and of the bicarbonate-dependent net flux. Human red cells were preincubated with varying concentrations of DIDS resulting in irreversible binding of between  $2.5 \times 10^6$  and  $1.2 \times 10^8$  DIDS molecules per cell. After the DIDS treatment one sample of cells was used for the determination of chloride self-exchange. The other sample was washed 3 times with 150 mM  $\text{NaHCO}_3$  at room temperature, which according to chemical analysis caused complete replacement of cellular chloride with bicarbonate. The cells were then suspended in 150 mM  $\text{NaHCO}_3$  medium and the  $\text{Na}$  transport as determined at  $38^\circ\text{C}$  and pH 7.4. The results (obtained with cells from two donors) demonstrated that the chloride transport and the bicarbonate-sensitive sodium net flux are equally reduced by DIDS (correlation coefficient  $r=0.994$ ). The bicarbonate-stimulated  $\text{Na}$  net flux was reduced from 16.4 to 2.5 mmol/kg cell solids at 100% inhibition.

from zero to 150 mM (substituting  $\text{Cl}^-$  with  $\text{HCO}_3^-$ ) increased the  $\text{Na}$  influx from 7 to 25 mmol/kg cell solids/h. A similar effect is seen on the net flux of  $\text{Na}$  into ouabain-treated cells. Table 1 shows that this effect of bicarbonate is completely eliminated if the red cells have been treated with the anion-reagent DIDS, which so far has proved to be a specific inhibitor of anion transport in erythrocytes. It is obvious that increase of bicarbonate concentration from zero to 150 mM has no effect on the influx of  $\text{Na}$  into DIDS-treated cells.

The quantitative relation between graded inhibition of bicarbonate-stimulated  $\text{Na}$  net flux and graded inhibition of the anion exchange system is shown in Fig. 1. There was identity between the fractional inhibition of the two transport processes. The quantitative effect of DIDS-binding to the red cell membrane on the bicarbonate-induced  $\text{Na}$  net flux was examined as shown in Fig. 2. It was found

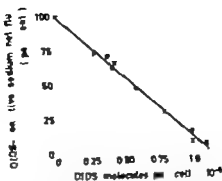


Fig. 2 The relation between the number of DIDS molecules bound per erythrocyte and the degree of inhibition of the DIDS-sensitive bicarbonate-stimulated  $\text{Na}$  net flux. (Regression line  $y = -89.4 + 100.9$  correlation coefficient 0.994.) Complete inhibition of the bicarbonate-induced sodium transport is obtained by the binding of  $1.13 \times 10^8$  DIDS molecules per cell. The results are obtained with cells from two donors.

that the inhibition of the  $\text{Na}$  transport bears the same quantitative relationship to the binding of DIDS molecules to the red cells as has previously been found for anion transport (Lepke et al. 1976; Slap et al. 1977; Funder et al. 1978). Binding of  $1.13 \times 10^8$  molecules per cell causes a complete abolition of the bicarbonate-stimulated  $\text{Na}$  net flux.

The anion transport in human red cells is characterized by a pronounced temperature dependence. The apparent Arrhenius activation energy of chloride transport is 20 kcal/mol (=84 kJ/mol) above  $15^\circ\text{C}$  (Brahm 1977). The temperature dependence of the bicarbonate-stimulated  $\text{Na}$  influx was examined in the temperature range between 25 and  $38^\circ\text{C}$ . It was found that the overall activation energy of the transport process was 20.8 kcal/mol (=87 kJ/mol) (Table 1). The apparent activation energy was somewhat lower in the DIDS-treated cells (17.0 kcal/mol) (=71 kJ/mol). The apparent activation energy of the DIDS-sensitive  $\text{Na}$  influx, calculated by difference, was 22.1 kcal/mol (=93 kJ/mol). It was not possible to extend the study to lower temperatures due to the pronounced decline of sodium transport with temperature.

#### The effect of bicarbonate on $\text{K}$ , $\text{Rb}$ and $\text{Cs}$ transport

Passive movements of  $\text{K}$ ,  $\text{Rb}$  and  $\text{Cs}$  were also studied in the presence of ouabain. It was found

Table 1 The effect of bicarbonate on fluxes of Na in normal and DIDS treated human erythrocytes at 7.4 35°C ouabain  $10^{-4}$  M

The media contained 150 mM Na<sup>+</sup> as the only cation. Chloride was substituted with bicarbonate keeping the anions 150 mM. The influx of Na ( $M_{in}$ , mmol/kg cell solids/h) without pretreatment of the cells with DIDS was a linear function of the bicarbonate concentration  $M_{in}=0.14 \times \text{HCO}_3^- + 5.7$  (correlation coefficient 0.99). In the DIDS treated cells the effect of bicarbonate was abolished and the permeability of the cell membrane to Na<sup>+</sup> was the same as in 150 mM NaCl medium. The results were obtained with erythrocytes from one donor and are the mean values of eight experiments at each experimental condition.

mM HCO <sub>3</sub> <sup>-</sup>	0 DIDS			+ DIDS		
	$M_{in}$ (mmol/kg cell solids/h)	$M_{out}$ (mmol/kg cell solids/h)	$M_{net}$ (mmol/kg cell solids/h)	$M_{in}$	$M_{out}$	$M_{net}$
0	6.9	4.2	2.7	6.1	3.9	2.2
50	10.0	4.0	6.0	5.9	3	2.7
100	18.2	7.9	10.3	5.8	6	3.2
150	24.8	8.1	16.7	5.8	2.6	3

Inhibition of chloride self-exchange and bicarbonate stimulated Na<sup>+</sup> net flux (Fig. 1 and 2) were done as described by Funder et al. (1978). The same reference gives information about the chemical data of the DIDS preparation. DIDS was stored in the dark at -20°C as a dry powder and a fresh solution was prepared for each experiment. In a few experiments it was found that potassium influx varied by a factor of two in cells that had been pretreated at a DIDS concentration above 10. DIDS molecules per cell. The potassium influxes in DIDS-treated cells were therefore studied after adding DIDS ( $2 \times 10^4$  molecules per cell) to the suspension 15 min before the experiment was started by the addition of <sup>42</sup>K. Control experiments showed that anion exchange is inhibited by more than 99% by this procedure.

<sup>86</sup>Rb (as RbCl 1.15 Ci/g Rb), <sup>137</sup>Cs (carrier free as CsCl) <sup>42</sup>K (as KCl 1.3 Ci/g K on delivery) from AEA, Risø, Denmark and <sup>22</sup>Na (as NaCl 900 Ci/g Na) were obtained from Radiochemical Centre, Amersham, England. <sup>137</sup>Cs was dissolved in 1 M HCl which was neutralized with CaOH before use. The isotopes were counted by their gamma radiation in a gamma well scintillation counter (Packard Modumate VI model 52-1) 0.1-0.2 µCi/ml cell suspension was used.

The flux studies were performed in 150 mM salt solutions of chloride or bicarbonate the cation being either Na<sup>+</sup>, K<sup>+</sup>, Rb<sup>+</sup> or Cs<sup>+</sup> (Table 4, Figs. 1 and 2). In the experiments with varying bicarbonate concentration (Tables 1 and 3) the media were prepared by mixing the NaCl and NaHCO<sub>3</sub>-solutions in the proportion giving the anion composition indicated in the Tables. pH in these media was kept at 7.4 by adjusting the CO<sub>2</sub> partial pressure to the appropriate value (550 mmHg in the 150 mM bicarbonate medium). In the 150 mM Na<sup>+</sup> media the concentration of ouabain was increased to 10<sup>-4</sup> M in order to ensure complete inhibition of the active potassium flux.

The unidirectional fluxes of sodium (Table 1) were calculated by means of an integrated flux equation based on the linear increase of cellular sodium content during the experiment as described by Funder & Welch (1967a).

Potassium in the cells and the media was determined by flame photometry. The net fluxes of potassium could therefore be calculated both from the loss of cell potassium and from the gain of potassium in the media. As the flux values calculated by the two methods were of the same magnitude the figures given in Table 3 are the mean values of the net flux calculated by the two methods. The influx of K<sup>+</sup>, Rb<sup>+</sup> and Cs<sup>+</sup> (Table 4) was calculated by means of the known extracellular specific activity of the cations and the uptake of the radioactivity over periods during which the radioactivity of the cell increased linearly with time (< 3 hours).

The mean apparent activation energy  $E_a$  in the temperature interval from 38 to 35°C (Table 2) was calculated from the equation (Eyring 1964)

$$E_a = \frac{RT_1T_2}{T_1 - T_2} (\ln J_1 - \ln J_2)$$

where  $T_1$  and  $T_2$  are the upper and lower absolute temperature,  $R$  is the gas constant and  $J_1$  and  $J_2$  are the Na<sup>+</sup> influxes at the two temperatures. All fluxes are expressed in units of mmol/kg cell solids/h. The fluxes can easily be related to membrane area, because  $3.1 \times 10^7$  cells, the number of normal cells containing one kg cell solid, have a membrane area of  $4.4 \times 10^4$  cm<sup>2</sup>.

## RESULTS

### The effect of the anion transport system for bicarbonate stimulated Na<sup>+</sup> transport

Unidirectional fluxes of Na<sup>+</sup> were studied in ouabain treated human red cells suspended in electrolyte media with increasing concentrations of bicarbonate. The results are shown in Table 1 and they confirm the effect of bicarbonate on Na<sup>+</sup> influx into ouabain treated cells (Funder & Welch 1967b). A gradual increase of bicarbonate concentration

4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)

which can be transported by the anion exchange mechanism. This ion pair hypothesis which has recently been reviewed by Lew & Beauge (1978) received support when Callaban & Goldstein (1972, 1978) found that the  $\text{HCO}_3^-$  induced  $\text{Na}^+$  influx is sensitive to 4-acetamido-4-isothiocyanato-stilbene sulfinic acid (SITS) a potent inhibitor of anion transport (Knauf & Rothstein 1971). Similar observations on the inhibitory effect of anion transport blockers on  $\text{HCO}_3^-$  stimulated  $\text{Li}^+$  fluxes were made by Duhm & Becker (1977). Funder et al. (1978) established the quantitative relation between inhibition of anion transport and of the  $\text{HCO}_3^-$  stimulated  $\text{Li}^+$  net flux. The inhibitor used was DIDS which so far is the inhibitor with the highest specificity for the transport system (Shap et al. 1977).

The purpose of the present study was to examine whether also the bicarbonate-stimulated sodium permeability can be correlated to the functional capacity of the anion transport system. Moreover it was examined whether bicarbonate affects the permeability to  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{Cs}^+$  which are not assumed to form ion pairs with carbonate.

#### Sodium transport

It seems likely that the increase of  $\text{Na}^+$  influx which is seen when chloride in media and cells is substituted with bicarbonate is caused by the transport of  $\text{NaCO}_3^-$ . The effects of DIDS treatment of the cell membrane on  $\text{Cl}^-$  self-exchange and on  $\text{Na}^+$  net flux (Fig. 1) clearly indicates that the DIDS-sensitive  $\text{Na}^+$  flux is linearly correlated to the inhibition of  $\text{Cl}^-$  self-exchange. A complete (i.e. 99%) inhibition of  $\text{Cl}^-$  self-exchange is accompanied by total abolition of the bicarbonate-stimulated  $\text{Na}^+$  influx. Funder et al. (1978) demonstrated that inhibition of bicarbonate-stimulated  $\text{Li}^+$  net flux is linearly related to DIDS-binding to the erythrocyte membrane. Fig. 2 demonstrates that the same relationship holds for inhibition of bicarbonate-stimulated  $\text{Na}^+$  net flux and it is therefore likely that both  $\text{Li}^+$  and  $\text{Na}^+$  can be transported by the anion exchange system in the presence of  $\text{HCO}_3^-$ .

One might anticipate that the transport of  $\text{NaCO}_3^-$  will vary with pH because the concentration of ion pairs varies linearly with carbonate concentration. However interpretation of the pH dependence of the bicarbonate-mediated  $\text{Na}^+$  transport is complicated by the variation of anion transport with pH. Thus  $\text{Cl}^-$  self-exchange in red blood

Table 4 Influx of  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{Cs}^+$  from 150 mM chloride or bicarbonate media into normal and DIDS-treated red cells

The amounts of DIDS used caused more than 99% inhibition of chloride self-exchange and of bicarbonate-induced  $\text{Na}^+$  and  $\text{Li}^+$  influx. The oxalate concentration was  $10^{-4}$  M in  $\text{Rb}^+$  and  $\text{Cs}^+$  media,  $10^{-5}$  M in the  $\text{K}^+$  media, pH 7.4, 38°C. At each experimental condition 6 experiments were performed with red blood cells from 3 donors.

150 mM	- DIDS (nmol/kg cell solids)	+ DIDS (h)
KCl	4.2 (3.0-5.5)	4.1 (4.3-3.7)
$\text{KHCO}_3$	4.4 (3.6-5.1)	3.3 (3.6-2.7)
RbCl	4.9 (6.0-4.1)	5.3 (6.3-4.6)
$\text{RbHCO}_3$	4.8 (3.4-4.3)	4.9 (6.1-3.7)
CsCl	4.3 (5.3-3.4)	4.5 (6.2-3.4)
$\text{CsHCO}_3$	4.3 (3.4-5.3)	4.0 (4.7-3.0)

cells decreases both above and below pH 7.4, where it has a well defined maximum at 38°C (Brahm 1977). The  $\text{HCO}_3^-$  stimulated  $\text{Na}^+$  influx has been found to double (from 10 to 22 nmol/kg cell solids/h) when pH is increased from 7.1 to 7.7 in a 142 mM  $\text{NaCO}_3^-$  medium (Wielth & Funder 1965). This pH variation causes a fourfold increase of  $\text{NaCO}_3^-$  concentration but at the same time anion exchange decreases by 30% between pH 7.2 and 7.8 (Brahm 1977) making a quantitative assessment difficult. It is impossible to examine  $\text{Na}^+$  influx at low pH values in the presence of 150 mM  $\text{HCO}_3^-$  because the pH is 6.94 when the medium is saturated with  $\text{CO}_2$  ( $P_{\text{CO}_2} = 720$  mmHg). It has therefore been very valuable that Becker & Duhm (1978) have reported that a number of oxyanions other than  $\text{CO}_3^{2-}$  appear to form ion pairs by a mechanism similar to that postulated for  $\text{CO}_3^{2-}$ . Because oxalic acid is a relatively strong acid with a pK of 3.8 the concentration of ion pairs does not vary in the interesting pH range between 6 and 7. Becker & Duhm (1978) found a 70% decrease of oxalate-induced  $\text{Li}^+$  uptake when pH was lowered from 7.4 to 6. Also in agreement with the studies of anion exchange in the red blood cells they found a slight decrease at pH values from 7.4 to 8.4 although it must be noted that the de-



Table 2. *Temperature dependence of Na<sup>+</sup> influx into normal and DIDS treated human erythrocytes suspended in 150 mM NaHCO<sub>3</sub> containing media (pH 7.4 ouabain 10<sup>-4</sup> M)*

The results for Na<sup>+</sup> influx are given as the mean values of 7 expts at each temperature with cells from one donor. The apparent activation energy is calculated as stated in the Methods section.

Temperature (°C)	Sodium Influx		
	(1) Untreated red cells (mmol/kg cell solids h <sup>-1</sup> )	(2) DIDS-treated red cells (h <sup>-1</sup> )	(1-2) DIDS-sensitive sodium influx
38	4.8	5.8	19.0
28	8.1	2.3	5.8
Apparent activation energy (kcal/mol)	20.8	17.2	22.1

active transport of the cations. The net loss of K<sup>+</sup> from red blood cells suspended in media containing 150 mM Na<sup>+</sup> and from zero to 150 mM HCO<sub>3</sub><sup>-</sup> (Cl<sup>-</sup> being the substituting anion) is shown in Table 3. There was no significant effect of increasing the HCO<sub>3</sub><sup>-</sup> concentration of the medium on the net loss of K<sup>+</sup>. At a concentration of zero mM HCO<sub>3</sub><sup>-</sup> the K<sup>+</sup> net flux was on an average 3.3 mmol/kg cell solids h<sup>-1</sup> to be compared with a net flux of 4.0 in the presence of 150 mM HCO<sub>3</sub><sup>-</sup> and this net flux was not reduced by DIDS treatment of the cells. The study also showed that there was no effect of DIDS on the K<sup>+</sup> loss at lower concentration of HCO<sub>3</sub><sup>-</sup>, evidenced by the fact that the K<sup>+</sup> net fluxes in the presence and absence of DIDS

at each HCO<sub>3</sub><sup>-</sup> concentration were almost identical.

Passive movements of K<sup>+</sup>, Rb<sup>+</sup> and Cs<sup>+</sup> were studied by incubating erythrocytes in 150 mM solutions of the chloride and bicarbonate salts of these three cations. Table 4 shows that there was a significant increase of the influx of the cations when 150 mM Cl<sup>-</sup> was substituted with 150 mM HCO<sub>3</sub><sup>-</sup>. It was also clear that DIDS treatment of the cells did not change the magnitude of Rb<sup>+</sup> and Cs<sup>+</sup> influx in Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> media. K<sup>+</sup> influx was unaffected by DIDS treatment in the Cl<sup>-</sup> medium. In a HCO<sub>3</sub><sup>-</sup> medium there was a reduction of the influx from 4.4 to 3.3 mmol/kg cell solids h<sup>-1</sup> after DIDS treatment of the cells and the K<sup>+</sup> influx in the DIDS-treated cells was also lower in the HCO<sub>3</sub><sup>-</sup> medium than in the Cl<sup>-</sup> medium.

Table 3. *The lacking of effect of bicarbonate on the net flux of K<sup>+</sup> from normal and DIDS treated erythrocytes incubated in media containing 150 mM Na<sup>+</sup> (pH 7.4 38°C ouabain 10<sup>-4</sup> M)*

Substitution of chloride with bicarbonate or treatment with DIDS did not change the permeability of the cell membrane to K<sup>+</sup>. The results were obtained from the experiments reported in Table 1.

mM HCO <sub>3</sub> <sup>-</sup>	0 DIDS M <sub>net</sub> (mmol/kg cell solids h <sup>-1</sup> )	+ DIDS M <sub>net</sub> (mmol/kg cell solids h <sup>-1</sup> )
0	3.3	9
50	3.7	3.8
100	4.6	4.9
150	4.0	4.0
Mean	3.9	3.9
S.D.	0.65	0.36
n	8	8

## DISCUSSION

Interest in the effect of HCO<sub>3</sub><sup>-</sup> on Na<sup>+</sup> transport through the erythrocyte membrane was prompted by the clinical observation that patients with metabolic alkalosis, characterized by an increased HCO<sub>3</sub><sup>-</sup> concentration in the plasma, accumulate Na<sup>+</sup> in their erythrocytes (Funder & Wieth 1974a, b). *In vitro* studies showed that the Na<sup>+</sup> uptake was caused by an increased passive permeability to Na<sup>+</sup> (Funder & Wieth 1967b) and that the active transport of Na<sup>+</sup> was not affected in the presence of HCO<sub>3</sub><sup>-</sup> (Wieth 1969). Wieth (1970) observed that both Li<sup>+</sup> and Na<sup>+</sup> permeabilities are affected and proposed that the increased permeabilities were due to the formation of the ion pairs LiCO<sub>3</sub><sup>-</sup> and NaCO<sub>3</sub><sup>-</sup>.

which can be transported by the anion exchange mechanism. This ion pair hypothesis which has recently been reviewed by Lew & Beauge (1978) received support when Callahan & Goldstein (1972-1978) found that the  $\text{HCO}_3^-$  induced  $\text{Na}^+$  influx is sensitive to 4-acetamidobenzyl-2-thiothiuronium-2-sulfonate (SITS), a potent inhibitor of anion transport (Knauf & Rothstein 1971). Similar observations on the inhibitory effect of anion transport inhibitors on  $\text{HCO}_3^-$  stimulated  $\text{Li}^+$  fluxes were made by Duhm & Becker (1977). Funder et al. (1978) established the quantitative relation between inhibition of anion transport and of the  $\text{HCO}_3^-$  stimulated  $\text{Li}^+$  net flux. The inhibitor used was DIDS which so far is the inhibitor with the highest specificity for the transport system (Schip et al. 1977).

The purpose of the present study was to examine whether also the bicarbonate-stimulated sodium permeability can be correlated to the functional capacity of the anion transport system. Moreover it was examined whether bicarbonate affects the permeability to  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{Cs}^+$  which are not assumed to form ion pairs with carbonate.

#### Sodium transport

It seems likely that the increase of  $\text{Na}^+$  influx which is seen when chloride medium and cells are substituted with bicarbonate is caused by the transport of  $\text{NaCO}_3^-$ . The effects of DIDS treatment of the cell membrane on  $\text{Cl}^-$  self-exchange and on  $\text{Na}^+$  net flux (Fig. 1) clearly indicates that the DIDS-sensitive  $\text{Na}^+$  flux is linearly correlated to the inhibition of  $\text{Cl}^-$  self-exchange. A complete (i.e. 99%) inhibition of  $\text{Cl}^-$  self-exchange is accompanied by total abolition of the bicarbonate-stimulated  $\text{Na}^+$  influx. Funder et al. (1978) demonstrated that inhibition of bicarbonate-stimulated  $\text{Li}^+$  net flux is linearly related to DIDS-binding to the erythrocyte membrane. Fig. 2 demonstrates that the same relation holds for inhibition of bicarbonate-stimulated  $\text{Na}^+$  net flux and it is, therefore, likely that both  $\text{Li}^+$  and  $\text{Na}^+$  can be transported by the anion exchange system in the presence of  $\text{HCO}_3^-$ .

One might anticipate that the transport of  $\text{NaCO}_3^-$  will vary with pH because the concentration of ion pairs varies linearly with carbonate concentration. However, interpretation of the pH dependence of the bicarbonate-mediated  $\text{Na}^+$  transport is complicated by the variation of anion transport with pH. Thus  $\text{Cl}^-$  self-exchange in red blood

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150 mM	0 DIDS (nmol/kg cell solids)	+ DIDS (h)
$\text{KCl}$	6.2 (5.0-3.5)	4.1 (4.3-5.7)
$\text{KHCO}_3$	4.4 (3.6-1)	3.3 (3.6-7)
$\text{RbCl}$	4.9 (6.0-1)	5.3 (6.3-4.6)
$\text{RbHCO}_3$	4.8 (5.4-3)	4.9 (6.1-3.7)
$\text{CsCl}$	4.1 (5.5-4)	4.5 (6-3.4)
$\text{CsHCO}_3$	4.3 (5.4-3.9)	4.0 (4.7-8)

cells decreases both above and below pH 7.2 where it has a well defined maximum at 38°C (Brahm 1977). The  $\text{HCO}_3^-$  stimulated  $\text{Na}^+$  influx has been found to double (from 10 to 22 nmol/kg cell solids/h) when pH is increased from 7.1 to 7.7 in a 142 mM  $\text{NaCO}_3^-$  medium (Wieth & Funder 1965). This pH variation causes a fourfold increase of  $\text{NaCO}_3^-$  concentration but at the same time anion exchange decreases by 30% between pH 7.4 and 7.8 (Brahm 1977) making a quantitative assessment difficult. It is impossible to examine  $\text{Na}^+$  influx at low pH values in the presence of 150 mM  $\text{HCO}_3^-$  because the pH is 6.94 when the medium is saturated with  $\text{CO}_2$  ( $P_{\text{CO}_2} = 720$  mmHg). It has therefore been very valuable that Becker & Duhm (1978) have reported that a number of oxyanions other than  $\text{CO}_3^{2-}$  appear to form ion pairs by a mechanism similar to that postulated for  $\text{CO}_3^{2-}$ . Because oxalic acid is a relatively strong acid with a  $\text{pK}_2$  of 3.8 the concentration of ion pairs does not vary in the interesting pH range between 6 and 7. Becker & Duhm (1978) found a 70% decrease of oxalate-induced  $\text{Li}^+$  uptake when pH was lowered from 7.4 to 6. Also in agreement with the studies of anion exchange in the red blood cells, they found a slight decrease at pH values from 7.4 to 8.4 although it must be noted that the de-

Table 5 The apparent association constants for the reaction between  $H^+$ ,  $Li^+$ ,  $Na^+$  and  $CO_3^{2-}$  (ionic strength 0.15 M)

	Association constant M
$H^+ + CO_3^{2-} \rightleftharpoons HCO_3^-$	$6.3 \times 10^6$ (1)
$Li^+ + CO_3^{2-} \rightleftharpoons LiCO_3^-$	$7.4 \times 10^1$ (2)
$Na^+ + CO_3^{2-} \rightleftharpoons NaCO_3^-$	$1.8 \times 10^1$ (3)

The values are taken from: (1) Siggaard-Andersen (1962) (2) Funder et al. (1978) (3) Garrels et al. (1961) Siggaard-Andersen (1962).

crease is considerably less steep than that found for  $Cl^-$  exchange at 38°C by Brahm (1977).

Table 5 shows estimates of the apparent association constants for association between  $H^+$ ,  $Li^+$ ,  $Na^+$  and  $CO_3^{2-}$ . The tendency of  $H^+$  to combine with  $CO_3^{2-}$  is  $10^6$  times greater than the tendency of  $Na^+$  and  $Li^+$  to form ion pairs with  $NaCO_3^-$ . However the concentration of  $Na^+$  and  $Li^+$  ion pairs in a 150 mM  $HCO_3^-$  solution at pH 7.4 will be high enough to explain the  $HCO_3^-$  induced increase of  $Na^+$  and  $Li^+$  fluxes found even if the permeabilities to the ion pairs are two orders of magnitude smaller than the  $Cl^-$  permeability (Funder et al. 1978).

#### Potassium transport

Garrels, Thompson & Siever (1961) and Garrels & Thompson (1962) concluded from electrometric determinations of activity coefficients in carbonate solutions that  $Na^+$  forms ion pairs with  $CO_3^{2-}$  to a significant degree, whereas ion pair formation between  $K^+$  and  $CO_3^{2-}$  could not be detected. Therefore the ion pair hypothesis predicts that the  $K^+$  permeability should not be affected by bicarbonate. The results of Table 3 clearly shows that there was no effect of  $HCO_3^-$  or of DIDS treatment of the cells on the  $K^+$  net flux from red cells into 150 mM  $Na^+$  media. The results of Table 4 further demonstrate that the  $K^+$  influx from media containing 150 mM  $K^+$  into red cells was not increased when  $Cl^-$  was replaced with  $HCO_3^-$ . However  $K^+$  influx from the bicarbonate medium decreased slightly but significantly by 1.1 mmol/kg cells solids/h in DIDS-treated cells. It can therefore not be excluded that a small amount of potassium can be transported as carbonate ion pairs. However the extra potassium influx is less than

10% of the bicarbonate-stimulated sodium influx shown in Table 1 so the amount transported as ion pairs must be very small. In this context it should be noted that Garrels & Thompson (1962) remark that the electrometric method would not be able to detect minor degrees of ion pair formation because one must accept deviations of up to  $10^4$  from the theoretical values of the activity coefficient. The present results may suggest that a small fraction of potassium influx is mediated by ion pair formation but the conclusion rests on the assumption that DIDS only affects the anion transport of the red cell membrane. Although DIDS has been found to be a highly specific inhibitor of anion transport it can of course not be excluded that the inhibitor may affect other membrane functions.

#### Rubidium and cesium transport

Robinson & Harned (1941) explained ion pair formation between oxyanions and alkali metal cations as a kind of ion solvent interaction where the oxygen atom of the anion replaces an oxygen atom in the hydration shell of the cation (localized hydrolysis). The tendency to ion pair formation decreases with increasing size of the cation predicting that ion pair formation will decrease through the series  $Li > Na > K > Rb > Cs$ . The hypothesis accordingly predicts that ion pair formation by  $Rb^+$  and  $Cs^+$  is even smaller than with  $K^+$ . In agreement with this prediction it was found that neither  $Rb^+$  nor  $Cs^+$  influx was affected by the presence of bicarbonate or by inhibition of the anion transport system with DIDS (Table 4).

#### Conclusion

The  $HCO_3^-$  dependent  $Na^+$  transport of the erythrocyte has been shown to be quantitatively related to the functional capacity of the anion transport system suggesting that  $Na^+$  like  $Li^+$  can be transported through the membrane in the form of carbonate ion pairs. The mechanism by which red cells incubated in a  $NaCO_3$  medium will take up  $Na^+$ ,  $HCO_3^-$  and water is identical to that shown by Funder et al. (Fig. 11) (1978) involving  $NaCO_3/HCO_3^-$  exchange through the anion pathway with a subsequent intracellular formation of  $HCO_3^-$  from  $CO_2$  diffusing into the cells (a mechanism similar to the so-called Jacobs-Stewart cycle first proposed by Jacobs & Stewart (1947)).

The findings add further support to the concept that the carbonate stimulation of cation fluxes is

specific to  $\text{Li}$  and  $\text{Na}$ . Bicarbonate has a negligible effect on potassium influx and no effect was found on the fluxes of  $\text{Rb}$  and  $\text{Cs}$ . It seems likely that this specificity can be explained by the theory of localized hydrolysis of Robinson & Harned (1941) because the electric field strength of the alkali metal cations with the larger crystal radii is too small to energize the formation of stable inter actions with carbonate ions.

I wish to thank Dr Jens Otto Wieth for many valuable discussions during the performance of this work.

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# Pre- and postjunctional beta-adrenoceptor mediated effects on transmitter release and effector response in the isolated rat portal vein

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DAHLÖF C., LJUNG B. & ÅBLAD B. Pre- and postjunctional beta-adrenoceptor mediated effects on transmitter release and effector response in the isolated rat portal vein. *Acta Physiol Scand* 1980; 108: 39-47. Received 27 April 1979. ISSN 0001-6772. Department of Pharmacology, AB Hansa, Mölndal, Sweden.

Pre- and postjunctional control mechanisms of the portal vein of spontaneously hypertensive rats (SHR) were characterized. Emphasis was placed on the influence of the presynaptic beta-adrenoceptor mediated mechanisms for regulation of neuronal noradrenaline (NA) release (studied as tractions overflow) and its consequences for the contractile response under *in vitro* conditions. It was found that isoprenaline increased, whereas *dl*-propranolol decreased the release of neuronal NA during transvenous nerve stimulation, while effector responses remained unaltered. *dl*-Propranolol and the beta-1 selective adrenoceptor antagonist, metoprolol, did not affect these two variables. It is concluded that the presynaptic beta-adrenoceptors in the rat portal vein are assembly of the beta-2 type and mediate facilitation of neuronal transmitter release and that concomitant changes of the effector responses of this tissue are below the level of detection under the present experimental conditions.

**Key words:** Aortic, portal vein, presynaptic beta-adrenoceptor, propranolol, metoprolol, transmitter release, effector response.

A variety of autacoids have been found to control the amount of transmitter released per impulse from adrenergic nerves (see Westfall 1977 for review). Noradrenaline appears to modulate its own release via two local feed-back mechanisms mediated via presynaptic adrenoceptors. Thus, prejunctional alpha-adrenoceptors mediate a negative feed-back mechanism (Furness & Hamburger 1971; Kirpekar & Pong 1971; Langer et al. 1971) whereas prejunctional beta-adrenoceptors mediate a positive feed-back control (Langer et al. 1974; Adler-Gaschinsky & Langer 1975). The major emphasis in most studies on prejunctional control mechanisms is placed on changes in transmitter release per se (see Starke 1977; Langer 1977) and less attention has been paid to the functional consequences for the effector response of altered amounts of noradrenaline (NA) being released per impulse.

In a previous study (Åblad et al. 1970; Dahlöf et

al. 1975) on the vascular resistance of the perfused hind limb of the cat we found that propranolol decreased NA release during sympathetic vasomotor nerve stimulation by up to 30% whereas the vasoconstrictor response was reduced by 15%.

The aim of the present study was to characterize adrenergic prejunctional control mechanisms in an isolated vascular preparation, the portal vein of spontaneously hypertensive rats (SHR). The main emphasis was given to the influence of the presynaptic beta-mediated mechanism for the regulation of neuronal NA release and its consequences for the contractile response.

Two types of experiments were performed. First, the effects of *dl*-propranolol on the contractile responses to transvenous nerve stimulation (TNS) at graded frequencies and to exogenous NA were quantitated. Second the portal veins were preincubated with <sup>3</sup>H 1-NA and the effects of various com-



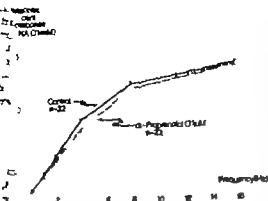


Fig. 1. Excitatory response of rat (SHR) portal vein to incremental nerve stimulation for 1 min at graded impulse rates. Values obtained before and after exposure to *dl*-propranolol ( $0.1 \mu\text{M}$ ). Responses expressed as per cent of maximal responses to exogenous NA ( $100 \mu\text{M}$ ). Means  $\pm$  S.E.  $n=22$ .

Statistical evaluation of the results was done by Student's *t*-test. Mean values were considered statistically significant when *P*-values are less than 0.05.

## RESULTS

### Effect of *dl*-propranolol on the contractile response to TNS at graded frequencies and to exogenous NA

The effector responses to TNS at graded frequencies expressed as a percentage of maximum response in exogenous NA ( $100 \mu\text{M}$ ) first studied in the control situation and subsequently during exposure to propranolol ( $0.1 \mu\text{M}$ ) are shown in Fig. 1. The maximum response to TNS before addition of propranolol was obtained at 16 Hz and amounted to  $91 \pm 1.4\%$  ( $n=22$ ) of that produced by exogenous NA ( $100 \mu\text{M}$ ). In presence of *dl*-propranolol ( $0.1 \mu\text{M}$ ) there was a tendency to a small shift of the frequency response curve to the right. However, a minimal shift to the right was also noted in the control experiments, where the second frequency response curve was obtained in normal Krebs solution without addition of propranolol. In order to quantitate an effect caused by *dl*-propranolol the changes in response to TNS at each frequency have been expressed as a percentage of the initial response to the same frequency. There was a significant reduction in effector response to TNS only at a frequency of 4 Hz during *dl*-propranolol exposure as compared to control experiments ( $-12 \pm 1.6$  and  $-6 \pm 1.9\%$  res-

pectively  $P < 0.05$ ). The maximal mean force response of the portal vein to exogenous NA ( $13.8 \pm 0.5 \text{ mN}$ ) was not affected by the exposure to *dl*-propranolol ( $0.1 \mu\text{M}$ ) nor was the maximum peak force value.

In the presence of cocaine exogenous NA in a concentration of  $0.03 \mu\text{M}$  produced a contractile response which corresponded to about 20% of the maximum response obtained by exogenous NA ( $100 \mu\text{M}$ ). The vasoconstrictor responses to exogenous NA did not differ in the two experimental groups neither before nor after one group had been treated with *dl*-propranolol ( $0.5 \mu\text{M}$ ).

### Effects on neuronal $\text{H}$ -release and contractile response to TNS at 2 Hz

Fig. 2 shows original tracings of contractile force and of integrated force and bars representing  $^3\text{H}$  overflow in consecutive 4 min samples from an experiment on isolated superfused SHR portal vein. In the control situation phasic contractions are seen with intervening periods of complete relaxation returning to the baseline of passive force. In response to TNS for 1 min at 4 Hz (S1 = "primer") and at 2 Hz (S2, S3) respectively the rate and amplitude of the contractions increased to form an incomplete tetanus. The concomitant increase in  $^3\text{H}$ -overflow is indicated by the stippled portion of the  $\text{H}$ -overflow bar. Test substances were introduced at the time indicated, i.e. 16 min prior to the beginning of S3. For details see Methods.

**Effects on transmitter release.** The average fractional  $\text{H}$ -release during S1 ranged between  $2.7 \pm 0.3 \times 10^{-6}$  ( $n=4$ ) and  $4.0 \pm 0.01 \times 10^{-6}$  ( $n=6$ ) in the various series of experiments. In Fig. 3a and b the effects of the tested substances on fractional  $\text{H}$ -release are expressed in terms of the response to S3 in comparison with that to S2 from individual values obtained in each series. In the control experiments the fractional  $\text{H}$ -release during S3 compared to that during S2 was reduced by  $7 \pm 3\%$  ( $n=9$ ). This change was taken into account when the statistical evaluation of the effects of the tested compounds was performed (see Methods).

Fig. 3 shows the effects of phenoxybenzamine (PBA,  $1 \mu\text{M}$ ) and tetrodotoxin (TTX,  $0.8 \mu\text{M}$ ). PBA elicited a marked increase of fractional  $\text{H}$ -release by  $473 \pm 53\%$  ( $P < 0.001$ ). After TTX on the other hand, the  $^3\text{H}$ -release response to TNS was practically abolished.

The beta-adrenoceptor active compounds



pounds on transmitter release were studied as fractional  $^3\text{H}$ -overflow and related to the simultaneous ly recorded contractile response during TNS

## MATERIAL AND METHODS

Spontaneously hypertensive rats (SHR) of the Okamoto strain (Møllegaard Hansens avlslaboratorier A/S Den mark) weighing 250–350 g and of either sex were used. Arterial blood pressure (BP) was not measured in the rats used in this study but SHR of corresponding size from the same supplier had mean BP of 150–180 mmHg when measured via indwelling catheters in conscious unrestrained animals (Ljung unpublished observations). The rats were sacrificed by decapitation. The portal vein was dissected free of surrounding tissue and incised to form a longitudinal strip which was tied at both ends with fine silk and mounted under 5 mN passive force in an organ bath for isometric recording of contractile force by means of a Grass FT 03 transducer on a Grass polygraph. The output signal was electronically integrated over 1 min periods and the integral mean force was recorded on a separate polygraph channel. An induced response was quantitated as the average mean force over the period of stimulation minus the average mean force developed spontaneously during the preceding 3 min control period. All tissues were allowed to equilibrate in the Krebs solution (see below) for 1 h before the experiment was started.

Transmural nerve stimulation was performed by applying square wave pulses of 0.8 ms duration and 700 mA current in alternate directions. This current was shown to be of supramaximal intensity in separate experiments where the contractile responses to graded currents were studied. The pulses were delivered by a constant current stimulator (Elg) to be published) between two electrodes consisting of rectangular sheets of platinum 7 mm apart placed on either side of the preparation. The current density of the field was estimated to be 400 mA/cm<sup>2</sup>. Due to the design of the biphasic stimulator electrolysis at the electrodes was minimized. The experimental procedure and protocol were as follows.

### Effects of propranolol on responses to TNS at graded impulse rates and to exogenous NA

Two portal vein preparations were mounted in the same organ bath. One min period of TNS were applied sequentially at 10 min intervals at the frequencies of 1, 4, 8 and 16 Hz respectively. After 30 min during which three rinses of the bath were performed the same sequence of stimulations was repeated. The effect of *dl*-propranolol 0.1  $\mu\text{M}$  on the frequency response relationship was tested in 22 preparations which were continuously exposed to the antagonist added to the bath solution 30 min prior to the beginning of the second set of TNS applications. The neurogenic effector response was expressed as percentage of the maximum response to exogenous NA 100  $\mu\text{M}$  added to the medium at the end of the experiment.

In one set of experiments the effect of *dl*-propranolol on responses to exogenous NA was analyzed. Cocaine (3  $\mu\text{M}$ ) was added to the Krebs solution during the accommodation period and maintained throughout the experi-

ments to minimize neuronal uptake. Noradrenaline 1  $\mu\text{M}$  was applied during the three min long exposure periods at 30 min intervals. Immediately after the NA exposure *dl*-propranolol (0.3  $\mu\text{M}$ ) was added to 1 of 8 tissues studied and kept in the solution during remainder of the experiment. Each response value expressed as a percentage of the average obtained at the initial three NA control responses.

### Pharmacological effects on transmitter release and effector response

The portal vein preparation was incubated for 30 s in Krebs solution containing 0.9  $\mu\text{M}$  HNA. It was rinsed four times with NA-free Krebs solution mounted in a 1 ml superfusion bath. During the subsequent 1 h wash-out period the preparation was perfused with Krebs solution at a rate of 1.5 ml/min. Initial stimulation period (S1) = 480 pulses at 4 Hz after the end of the HNA incubation was used as "condition" the tissue and the H output and effector responses were disregarded. The second and third stimulation period (S2 and S3 = 40 shocks at 4 Hz) were applied 4 and 48 min after S1 respectively. The activities of the superfusate fractions collected over 4 min periods and of the tissue content after digestion in 1 ml Soluene 100 (Packard Instr.) were determined by counter in 10 ml Dimulene (Packard Instr.) in a Packard Tricarb Scintillation Spectrometer. The fractional overflow per impulse was calculated as:

$$\text{H efflux induced by stimulation} = \frac{\text{stimulation} - \text{spontaneous \% of}}{\text{Total H in tissue at the beginning of stimulation}} \times \frac{\text{Number of stimulation impulses}}{\text{impulses}}$$

Values for H release and effector response during the third stimulation period (S3) were expressed as a percentage of those obtained during the second stimulation period (S2). The average percentage control values obtained during S3 served as a base for the statistical evaluation of drug induced changes of responses to S1. Various substances tested were added to the perfusion medium 16 min before S3. Results are presented as mean  $\pm$  S.E.

### Solution and drugs

The modified Krebs solution used in all experiments had the following composition in mM: NaCl 122, KCl 4, CaCl<sub>2</sub> 3.0, MgCl<sub>2</sub> 1.19, NaHCO<sub>3</sub> 15.5, KH<sub>2</sub>PO<sub>4</sub> 1.19, glucose 11.5 and CaNa<sub>2</sub> versenate 0.26. It was bubbled 3% CO<sub>2</sub> in O<sub>2</sub> giving a pH of 7.4. The temperature was kept at 38°C. The following drugs were used.

Noradrenaline (1-arterenol bitartrate, Sigma Chemical Co.) 1 H-noradrenaline specific activity 5.85 Ci/mmol (NEN), isoprenaline hydrochloride, *dl*-propranolol hydrochloride and *dl*-propranolol hydrochloride (ICI), *dl*-methoxypropranolol bitartrate (ICI), phenylephrine hydrochloride (SK & F), tetrodotoxin (Sigma Chemical Co). Stock solutions 0.01 M HCl or saline were diluted in Krebs solution immediately prior to administration.

— Control  
— 1.1  $\mu$ M PBA  
— 1.1  $\mu$ M TTX

spectively  $P < 0.05$ ). The maximal mean force response of the portal vein to exogenous NA ( $13.8 \pm 0.5$  mN) was not affected by the exposure to *dl*-propranolol ( $0.1 \mu$ M) nor was the maximum peak force value.

In the presence of cocaine, exogenous NA in a concentration of  $0.03 \mu$ M produced a contractile response which corresponded to about 20% of the maximum response obtained by exogenous NA ( $100 \mu$ M). The vasoconstrictor responses to exogenous NA did not differ in the two experimental groups neither before nor after one group had been treated with *dl*-propranolol ( $0.3 \mu$ M).

#### Effects on neuronal $^3$ H-release and contractile response to TNS

Fig. 2 shows original tracings of contractile force and of integrated force and bars representing  $^3$ H-overflow in consecutive 4 min samples from an experiment on isolated superfused SHR portal vein. In the control situation phasic contractions are seen with intervening periods of complete relaxation returning to the baseline of passive force. In response to TNS (for 2 min at 4 Hz (S1 = primer) and at 2 Hz (S2-S3) respectively) the rate and amplitude of the contractions increased in form an incomplete tetanus. The concomitant increase in  $^3$ H-overflow is indicated by the stippled portion of the  $^3$ H-overflow bar. Test substances were introduced at the time indicated, i.e. 16 min prior to the beginning of S3. For details see Methods.

**Effects on transmitter release.** The average fractional  $^3$ H-release during S2 ranged between  $2.7 \pm 0.3 \times 10^{-6}$  ( $n=4$ ) and  $4.0 \pm 0.01 \times 10^{-6}$  ( $n=6$ ) in the various series of experiments. In Fig. 3a and b the effects of the tested substances on fractional  $^3$ H-release are expressed in terms of the response to S3 in comparison with that to S2 from individual values obtained in each series. In the control experiments the fractional  $^3$ H-release during S3 compared to that during S2 was reduced by  $7 \pm 3\%$  ( $n=9$ ). This change was taken into account when the statistical evaluation of the effects of the tested compounds was performed (see Methods).

Fig. 3a shows the effects of phenoxybenzamine (PBA  $1 \mu$ M) and tetrodotoxin (TTX  $0.1 \mu$ M). PBA elicited a marked increase of fractional  $^3$ H-release by  $473 \pm 53\%$  ( $P < 0.001$ ). After TTX on the other hand the  $^3$ H-release response to TNS was practically abolished.

The beta-adrenoceptor active compounds

Fig. 1. Excitatory response of rat (SHR) portal vein to nociceptor nerve stimulation for 1 min at graded impulse rates. Values obtained before and after exposure to *dl*-propranolol ( $0.1 \mu$ M). Responses expressed as per cent of maximal responses to exogenous NA ( $100 \mu$ M). Means  $\pm$  S.E.  $n=22$ .

#### Methods

Statistical evaluation of the results was done by Student's *t*-test. Mean values were considered statistically significant when  $P$  values were less than 0.05.

## RESULTS

### Effect of *dl*-propranolol on the contractile response to TNS at graded frequencies and to exogenous NA

The effector responses to TNS at graded frequencies, expressed as a percentage of maximum response to exogenous NA ( $100 \mu$ M) first studied in the control situation and subsequently during exposure to propranolol ( $0.1 \mu$ M) are shown in Fig. 1. The maximum response to TNS before addition of propranolol was obtained at 16 Hz and amounted to  $91 \pm 1\%$  ( $n=22$ ) of that produced by exogenous NA ( $100 \mu$ M). In presence of *dl*-propranolol ( $0.1 \mu$ M) there was a tendency to a small shift of the frequency response curve to the right. However, a minimal shift to the right was also noted in the control experiments, where the second frequency response curve was obtained in normal Krebs solution without addition of propranolol. In order to quantitate an effect caused by *dl*-propranolol the changes in response to TNS at each frequency have been expressed as a percentage of the initial response to the same frequency. There was a significant reduction in effector response to TNS only at a frequency of 4 Hz during *dl*-propranolol exposure as compared to control experiments ( $-12 \pm 1.6$  and  $-6 \pm 1.9\%$  re-

pounds on transmitter release were studied as fractional  $^3\text{H}$ -overflow and related to the simultaneously recorded contractile response during TNS

## MATERIAL AND METHODS

Spontaneously hypertensive rats (SHR) of the Okamoto strain (Møllegaard Hansens avlslaboratorier A/S Denmark) weighing 250–350 g and of either sex were used. Arterial blood pressure (BP) was not measured in the rats used in this study but SHR of corresponding size from the same supplier had mean BP of 150–180 mmHg when measured via indwelling catheters in conscious unrestrained animals (Ljung unpublished observations). The rats were sacrificed by decapitation. The portal vein was dissected free of surrounding tissue and incised to form a longitudinal strip which was tied at both ends with fine silk and mounted under 5 mN passive force in an organ bath for isometric recording of contractile force by means of a Grass FT 03 transducer on a Grass polygraph. The output signal was electronically integrated over 1 min periods and the integral mean force was recorded on a separate polygraph channel. An induced response was quantitated as the average mean force over the period of stimulation minus the average mean force developed spontaneously during the preceding 3 min control period. All tissues were allowed to equilibrate in the Krebs solution (see below) for 1 h before the experiment was started.

Transmural nerve stimulation was performed by applying square wave pulses of 0.8 ms duration and 200 mA current in alternate directions. This current was shown to be of supramaximum intensity in separate experiments where the contractile responses to graded currents were studied. The pulses were delivered by a constant current stimulator (Elg to be published) between two electrodes consisting of rectangular sheets of platinum 7 mm apart placed on either side of the preparation. The current density of the field was estimated to be 400 mA/cm<sup>2</sup>. Due to the design of the biphasic stimulator electrolysis at the electrodes was minimized. The experimental procedure and protocol were as follows.

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Two portal vein preparations were mounted in the same organ bath. One min periods of TNS were applied sequentially at 10 min intervals at the frequencies of 1, 4, 8 and 16 Hz respectively. After 30 min during which three rimers of the bath were performed the same sequence of stimulations was repeated. The effect of *dl*-propranolol (1  $\mu\text{M}$ ) on the frequency response relationship was tested in 22 preparations which were continuously exposed to the antagonist added to the bath solution 20 min prior to the beginning of the second set of TNS applications. The neurogenic effector response was expressed as a percentage of the maximum response to exogenous NA (100  $\mu\text{M}$ ) added to the medium at the end of the experiment.

In one set of experiments the effect of *dl*-propranolol on responses to exogenous NA was analyzed. Cocaine (3  $\mu\text{M}$ ) was added to the Krebs solution during the accommodation period and maintained throughout the experi-

ments to minimize neuronal uptake. Noradrenaline (1  $\mu\text{M}$ ) was applied during six three min long exposure periods at 30 min intervals. Immediately after the NA exposure *dl*-propranolol (0.5  $\mu\text{M}$ ) was added to 8 tissues studied and kept in the solution during remainder of the experiment. Each response was expressed as a percentage of the average obtained in the initial three NA control responses.

### Pharmacological effects on transmitter release and effector response

The portal vein preparation was incubated for 30 min in Krebs solution containing 0.9  $\mu\text{M}$  H. NA was rinsed four times with NA free Krebs solution mounted in a 1 ml superfusion bath. During the subsequent 1 h wash-out period the preparation was perfused with Krebs solution at a rate of 1.5 ml/min. Initial stimulation period (S1 = 480 pulses at 4 Hz) after the end of the H. NA incubation was used only condition the tissue and the H. output and effector responses were disregarded. The second and third stimulation periods (S2 and S3 = 40 shocks at 4 Hz) were applied 4 and 48 min after S1 respectively. The activities of the superfusate fractions collected over 4 min periods and of the tissue content after digestion with Soluene 100 (Packard Instr.) were determined by counting in 10 ml Dimulene (Packard Instr.) in a Packard LS Scintillation Spectrometer. The fractional overflow per impulse was calculated as

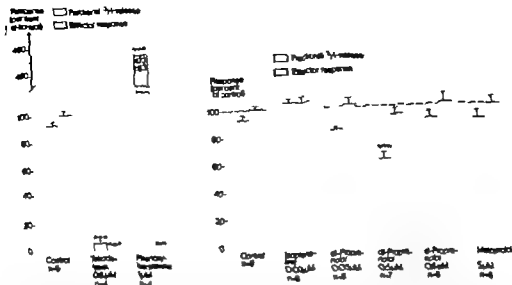
$$\text{H efflux induced by stimulation} = \frac{\text{spontaneous H efflux}}{\text{Total H in tissue at the beginning of stimulation}} \times \frac{\text{Number of stimulus impulses}}{\text{impulses}}$$

Values for H release and effector response during a third stimulation period (S3) were expressed as a percentage of those obtained during the second stimulation period (S2). The average percentage control obtained during S3 served as a base for the statistical evaluation of drug induced changes of responses to S3. Various substances tested were added to the perfusion medium 16 min before S3. Results are presented in mean  $\pm$  S.E.

### Solution and drug

The modified Krebs solution used in all experiments had the following composition in mM: NaCl 122, KCl 4, CaCl<sub>2</sub> 50, MgCl<sub>2</sub> 1.9, NaHCO<sub>3</sub> 15.5, KH<sub>2</sub>PO<sub>4</sub> 1.9, glucose 11.5 and CaNa<sub>2</sub> versenate 0.026. It was bubbled with CO<sub>2</sub> in O giving pH of 7.4. The temperature was kept at 38°C. The following drugs were used:

Noradrenaline (1-arterenol bitartrate (Sigma Chemical Co.) 1-H-noradrenaline pectate 5.83 Ci x mmol (NEN), isoprenaline hydrochloride, *dl*-propranolol hydrochloride and *dl*-propranolol hydrochloride (ICI), *dl*-isoproterenol bitartrate (H. S. de), phenoxylbenzamine hydrochloride (SK & F), tetrodotoxin (Sigma Chemical Co.) Stock solutions in 0.01 M HCl or saline were diluted in Krebs solution immediately prior to administration.



1 Effects of tetrodotoxin and phenoxybenzamine on transmitter release (open columns) and effector response (stippled columns) of the isolated rat (SHR) portal vein during TMS (53) at 2 Hz. \*\* indicates  $P < 0.001$  when values were compared to corresponding values in control tissues during 53.

2 Effects of propranolol, dl-propranolol and metoprolol on the transmitter release (open columns) and effector response (stippled columns) of the isolated rat (SHR) portal vein during TMS (53) at 2 Hz. \*\*\* indicates  $P < 0.05$  and 001 respectively when values were compared to corresponding values in control tissue during 53.

## DISCUSSION

Responses of the longitudinal smooth muscle of SHR portal vein to transmural field stimulation graded impulse rates resembled closely those previously described for portal vein preparations in normotensive rats of the Sprague-Dawley strain (Ljung 1970; Johansson et al. 1972). Furthermore the excitatory responses to transmural field stimulation were abolished by PBA or tetrodotoxin, which demonstrates that responses were selectively elicited via excitation of the intramural adrenergic nerve supply without any direct stimulation of the effector tissue. After PBA weak inhibitory responses to nerve activation at 2 Hz were sometimes noted which might indicate activation of postjunctional beta-adrenoceptors by NA released from the nerves. However neither the effector response to exogenous NA in a low dose (0.03  $\mu\text{M}$ ) nor the maximum response to exogenous NA (100  $\mu\text{M}$ ) were potentiated by beta-adrenoceptor blockade, which indicates that the alpha-adrenoceptor excitatory neurogenic control of the portal vein of the SHR is hardly influenced by postjunctional beta-mediated effects of NA.

Evoled overflow of tritium after  $^3\text{H}$  NA preincubation was used to estimate transmitter release. It

has previously been shown (Häggendal et al. 1970) that in the rat portal vein preparation about 85% of the increase in total tritium overflow induced by TNS at 4 Hz is practically intact  $^3\text{H}$  NA and that the remainder represents metabolites which in part have been formed after  $^3\text{H}$  NA release. In the present study there was no evidence for a tetrodotoxin-insensitive release of  $^3\text{H}$ -NA of neuronal and/or extraneuronal origin as has been found in other tissues (Schroff & Nedergaard 1975). It has not been established to what degree  $^3\text{H}$ -NA-release reflects the neuronal NA release of the portal vein. However in experiments in the perfused cat's spleen a proportional release of NA,  $^3\text{H}$  NA and dopamine-beta-hydroxylase occurred over a wide range of transmitter release elicited by low frequency nerve stimulation (Cubeddu et al. 1974). Altogether the discussed observations seem to validate the conclusion that alterations in relative  $^3\text{H}$  overflow reflect changes in total transmitter release from the adrenergic vasomotor nerves in response to nerve stimulation. This conclusion will be adopted later when the fractional  $^3\text{H}$ -release in the rat portal vein is discussed.

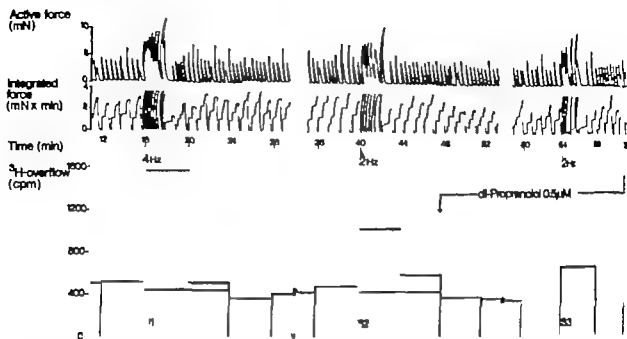


Fig 2 Original tracings of contractile force (upper panel) integrated force (middle panel) and column flow rate representing  $^3\text{H}$ -overflow in consecutive 4 min samples from an experiment on isolated superfused rat (S11R) portal vein with *dl* propranolol. Open columns represent the spontaneous overflow of radioactivity and the stippled peaks indicate increase in overflow above the resting levels produced by TNS. The first stimulation period (S1) was used as condition the tissue.

caused much less dramatic effects on fractional  $^3\text{H}$  release (Fig 3b). Addition of isoprenaline (ISO  $0.03 \mu\text{M}$ ) resulted in a small but statistically significant increase of fractional  $^3\text{H}$  release by  $12 \pm 3\%$  ( $P < 0.05$ ). Treatment with *dl* propranolol was on the other hand found to decrease the fractional  $^3\text{H}$  release the reduction being  $9 \pm 2\%$  ( $P < 0.05$ ) after  $0.05 \mu\text{M}$  and  $31 \pm 5\%$  ( $P < 0.001$ ) after  $0.5 \mu\text{M}$  *dl* propranolol. In contrast to this significant effect of  $0.5 \mu\text{M}$  *dl* propranolol, no effect on fractional  $^3\text{H}$ -release was observed after addition of the same concentration of *d* propranolol. Neither was the beta-1 selective antagonist metoprolol ( $5 \mu\text{M}$ ) found to affect the  $^3\text{H}$ -release response to TNS.

The spontaneous release of tritium from the portal vein preparation was not affected by the different substances tested except for a 3-fold increase produced by NA ( $100 \mu\text{M}$ ).

**Effects on effector response.** Control TNS during S2 evoked a contractile response corresponding to  $27 \pm 7\%$  ( $n = 38$ ) of the maximal response to exogenous NA ( $100 \mu\text{M}$ ), the mean values in the different experimental series varying from  $26 \pm 2\%$  ( $n = 6$ ) to  $28 \pm 2\%$  ( $n = 8$ ). In the control experiments the effector response during S3 did not differ from that obtained during S2 (Fig 3a and b).

Fig 3a shows that PBA ( $1 \mu\text{M}$ ) and TTX ( $1 \mu\text{M}$ ) completely abolished the effector response to TNS. A slight inhibitory response to TNS was observed in 3 of the 11 preparations treated with PBA, the reduction of integrated force being less than 5%.

Fig 3b shows how isoprenaline and the beta adrenoceptor antagonists influenced the effector response to TNS. No significant effect was observed after isoprenaline ( $0.03 \mu\text{M}$ ), *dl*-propranolol ( $0.05 \mu\text{M}$ ), *d*-propranolol ( $0.5 \mu\text{M}$ ) or metoprolol ( $5 \mu\text{M}$ ). After higher concentrations of *dl* propranolol ( $0.5 \mu\text{M}$ ) however, a tendency to a reduction ( $P = 0.082$ ) of the effector response to TNS was obtained.

The spontaneous activity of the portal vein was inhibited by ISO and after PBA treatment by exogenous NA ( $100 \mu\text{M}$ ). These inhibitory responses were characterized by an increased rate of spontaneous contraction whereas the amplitude of contraction was reduced to a relatively greater extent. The inhibition of the spontaneous activity caused by ISO ( $0.03 \mu\text{M}$ ) led to a reduction of integrated force by  $70 \pm 4\%$  ( $P < 0.01$ ).

Neither ISO nor the beta adrenoceptor blocker in the doses tested affected the maximum response to exogenous NA ( $100 \mu\text{M}$ ).

use in vascular resistance would only correspond to a 2% change in smooth muscle cell shortening according to the law of Poiseuille. A change of this order would be below the threshold of resolution in most *in vitro* conditions whether the vasoconstrictor responses are measured isometrically or tonometrically.

Attempts to classify the prejunctional beta-adrenoceptors according to the beta-1/beta-2 terminology of Lands et al (1967) have yielded varying results. In the meso limb of the cat the comparative effects of propranolol and the beta-1 selective antagonist metoprolol indicated that the presynaptic regulation of NA release was mainly mediated by beta-1 receptors (Dahlöf et al. 1974). However, the results obtained in studies on isolated blood vessels by Sjöström & Brundin (1976) and Westfall et al. (1978) indicate that the presynaptic receptors were of beta-2 type. This conclusion is supported by present findings with the beta-1 selective antagonist metoprolol. This compound did not affect norepinephrine release during TNS when given in a concentration producing marked inhibition of the basic chronotropic response to ISO in the isolated atrium (Johansson 1973). We have furthermore performed experiments on SHR portal vein with a series of beta-1 and beta-2 selective agonists and antagonists which confirm the existence of prejunctional receptors of the beta-2 type (Dahlöf *in press*). Thus in contrast to the results obtained in the anesthetized cat all discussed *in vivo* findings indicate that mainly beta-2 adrenoceptors are involved in the mediation of the facilitation of NA release. One possible interpretation of these data is that the positive control of neuronal release is mediated by presynaptic adrenoceptors of both the beta-1 and beta-2 type with variations regarding the relative importance of either receptor subtype in different species and tissues studied. This type of subreceptor arrangement was first suggested by Carlsson et al. (1972) to characterize postjunctional beta-adrenoceptor mediated effects and the hypothesis is supported by results from studies of cardiac stimulation (Carlsson et al. 1972), tracheobronchial smooth muscle relaxation (Furchgott 1976; Zaugg & Dahlöf 1976) and adipose tissue lipolysis (Belfrage et al. 1978). One factor of interest in this context is the differential beta-adrenoceptor affinity patterns of the agonists NA and A. Since NA has much higher affinity to beta-1 than to beta-2 adrenoceptors and

A has relatively more affinity to beta-2 than to beta-1 adrenoceptors, the two catecholamines may elicit a given beta-adrenoceptor mediated effect via different subreceptors (cf. Carlsson et al. 1972). This may be of interest when considering physiological aspects of hormonal and local neuronal control of presynaptic beta-adrenoceptor mediated feedback mechanism.

As regards a hormonal control Sjöström & Brundin (1973) first showed that presynaptic beta-adrenoceptors could be activated by adrenaline at concentrations similar to those found in plasma in man (cf. Vendsø 1960). In experiments made on the isolated SHR portal vein we found A to be a far more potent agonist for the presynaptic beta-adrenoceptor mechanism than NA (Dahlöf et al. 1978b). This finding supports the suggestion made by Sjöström et al. (1973) that beta-2 adrenoceptors are involved in a physiological control mechanism, through which circulating adrenaline facilitates the release of neuronal NA.

Regarding the local neuronal control of the presynaptic beta-adrenoceptor mediated mechanism, the NA released may be a significant modulator in certain neuroeffector systems as discussed above. However, the presynaptic alpha-adrenoceptor mediated feedback mechanism seems to be the major path by which neurally released NA modulates its own release (Strömberg 1977). The physiological importance of the local beta-adrenoceptor mediated positive feedback mechanism remains unclear. It has recently been suggested that the adrenergic neuron contains certain amounts of A which is released as a co-transmitter during nerve stimulation to act as agonist to presynaptic beta-adrenoceptors (Rand et al. 1978). Further studies are needed to clarify whether circulating and locally released A may serve to reinforce the regional effector control of the adrenergic nerve discharge.

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The fractional transmitter release per impulse in the SHR portal vein determined in the present experiments at 2 Hz was  $(3.4 \pm 0.3) \times 10^{-6}$ . A similar value was found in portal vein preparations from normotensive rats of the Sprague Dawley strain (Häggendal et al 1970). In a study by Pegram & Ljung (to be published) fractional  $^3\text{H}$  NA release in portal veins from SHR and from matched normotensive Wistar Kyoto rats was found not to differ. The available data thus indicate that fractional  $^3\text{H}$  release in the SHR portal vein does not differ from that obtained in normotensive rats.

PBA treatment caused an almost 5-fold increase of fractional  $^3\text{H}$  release. This effect was apparently due to blockade of the alpha-adrenoceptor mediated negative feedback control of transmitter release. However, PBA induced inhibition of neuronal and extraneuronal NA uptake mechanisms might be of contributory importance (Starke 1977). The PBA induced increase of transmitter release during TNS at 2 Hz was more pronounced than that obtained in previous experiments where the same experimental conditions but a lower stimulation frequency (1 Hz) was used (Dahlöf et al 1978a). This difference in transmitter release might indicate a frequency dependence of the presynaptic alpha-adrenoceptor mediated autoinhibition (cf. Westfall 1977).

In the present study *dl* propranolol ( $0.5 \mu\text{M}$ ) was found to reduce the fractional  $^3\text{H}$ -release during TNS by 31% while ISO caused an increase of the fractional  $^3\text{H}$  release by 12% in the SHR portal vein. The facilitating effect of ISO is somewhat lower than the increase we obtained by ISO in our previous study where the SHR portal vein was pretreated with PBA, desipramine and normetanephrine. In those experiments ISO in a comparable dose was found to increase the transmitter release by 25% and was at least as potent as adrenaline (A) (Dahlöf et al 1978b). The discrepancy is best explained by the tenet that the increase of transmitter release caused by ISO in the present experiments was counteracted by the presynaptic alpha-adrenoceptor mediated autoinhibition. *d* Propranolol, in contrast to *dl* propranolol in the same concentration, did not influence the fractional  $^3\text{H}$  release to TNS. It has been shown that *d*-propranolol exerts the same membrane stabilizing activity but is about 50 times less active as regards beta-adrenoceptor antagonism than *dl* propranolol (Barrett & Nunn 1970). We therefore conclude that the effect of *dl*-propranolol on fractional  $^3\text{H}$ -release

during TNS is due to inhibition of a presynaptic beta-adrenoceptor mediated positive feedback mechanism which was first suggested by Lar, al (1974). We have recently obtained more support for this concept in the portal vein preparation where pre- and postsynaptic alpha-adrenoceptor mediated functions as well as neuronal and extraneuronal NA-uptake mechanisms were inhibited (Dahlöf et al 1978a). Under such conditions endogenous NA was found to increase the fractional  $^3\text{H}$  release during TNS at 1 Hz and this effect was inhibited by *dl* propranolol which also reduced the fractional  $^3\text{H}$  release.

In spite of the clear cut reduction of fractional  $^3\text{H}$  release in the portal vein after *dl*-propranolol, very slight reductions of the effector response during TNS were obtained. This apparent discrepancy between transmitter release and effector response may be due to the fact that in the portal vein a nerve impulse seems to lead to very high and shortlasting local transmitter concentrations (Johansson et al 1972). It is an expected consequence of the high local concentrations to be associated with short neurotransmitter separation in the rat portal vein (Booz 1971) that variation of transmitter release per impulse and thus of the local transmitter concentration peak, even by a factor of two would affect the effector response only to a minimal extent (Ljung 1976). Furthermore, it is concluded above that the excitatory neurogenic control of the portal vein of the SHR is not affected by postjunctional beta mediated effects. Hence, it is not likely that a possible attenuation of the effector response due to reduced transmitter release would have been counterbalanced by simultaneous antagonism of beta mediated vasodilator response.

Evidence of a presynaptic beta-adrenoceptor mediated positive feedback mechanism operating *in vivo* was obtained in a previous study on the fused hind limb of the cat by Dahlöf et al (1978) that study *dl* propranolol decreased  $^3\text{H}$  NA release to sympathetic nerve stimulation by about 30%. This effect was associated with a consistent reduction of the vasoconstrictor response by about 10%. These findings could indicate that wider neurovascular separations prevail in those sections of vasculature which subserve the main resistance to the blood perfusion and that these sections possess a greater sensitivity to changes in transmitter release than the neuromuscular junctions in the SHR portal vein. On the other hand it may be argued that

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# Firing behaviour in stochastic nerve membrane models with different pore densities

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A stochastic nerve membrane model (a two-state pore system) was investigated by computer simulation in the uniform (space-clamped) case. The model was based upon the Hodgkin-Huxley equations for the giant axon in squid, but where both the maximal membrane conductances and the rate constants were changed systematically. This was done in order to simulate nerve membranes of small axons where both of these parameters are smaller than in squid. It was found that the effects upon the firing behaviour due to a finite number of pores were not greatly affected by changes in these parameters. When the specific injected current was calculated relative to the maximal membrane conductances, the threshold for firing was increased somewhat, and the frequency-current relationship became slightly more linear where the maximal conductances (or pore density) were decreased, or the rate constants increased. In the discussion it is shown how the results obtained could be applied qualitatively to the firing behaviour of nerve cells, and that firing in small nerve cells should be significantly influenced by the stochastic effects of finite number of pores. Output currents are also discussed, and their effects were found to be insignificant in small nerve cells.

In our former paper the firing behaviour of an isolated patch of nerve membrane (the space-clamped case) has been investigated when the effects of stochastic fluctuations of the membrane conductances due to a two-state pore system were taken into account (Skaugen & Walløe 1979). The equations used for the model membrane were based upon the equations empirically found for the squid giant axon by Hodgkin & Huxley (the H-H equations) (1951). All the membrane parameters including the maximal specific conductances were given the values used in the H-H equations.

It is only in very small nerve cells, however, that we could expect the effects of these stochastic fluctuations to influence the firing behaviour significantly. It is uniformly found that in small nerve fibres the density of sodium pores is much smaller than in the squid giant axon (Jack 1975). This probably also gives smaller values of the maximal specific membrane conductances. The effect of this may be at least partly counterbalanced by smaller rate constants, as will be shown here.

In this paper the effects upon the firing behaviour of different values of the maximal specific membrane conductances and the rate constants are investigated. The results are compared to those presented in the former paper and to the original non-stochastic H-H equations.

At the end of the paper the possible applications of the results obtained here and in the former paper are discussed. The possible effects of gating currents are also considered.

## THEORY

The simplest changes of some of the most important constants in the H-H equations at least from a computational point of view are to multiply all the membrane specific conductances ( $g_{Na}$ ,  $g_K$  and  $g_{Cl}$ ) by a factor  $\eta$  and to multiply all rate constants  $\alpha$ ,  $\beta$  by a factor  $\delta$  ( $\delta = k/m$  and  $n$ ). This is done in the "dimensional analysis" developed by Huxley in order to find how the conduction speed depended upon changes in these parameters (Huxley 1959).



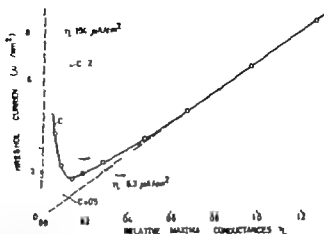


Fig. 1. Threshold current as function of the relative maximal membrane conductances for different values of the membrane capacitance  $C$ .  $C = 0$  —  $C = 1$   $\mu\text{F}/\text{cm}^2$ .  $C = 0.5$  and  $\mu\text{F}/\text{cm}^2$ .

er (above threshold). As the stochastic equations are not solved for  $\eta$  smaller than 0.1 this was not considered important.

The lower broken line in the figure, given by  $\eta = 6.3 \mu\text{A}/\text{cm}^2$  shows the threshold current in the case of zero membrane capacitance. Inspection of Eq. (1) shows that, except for the injected current, an increase in  $\eta = \eta_0/\eta_1$  is equivalent to a decrease in  $C$ . For  $\eta = 1$  the threshold current will steadily approach  $\approx 6.3 \mu\text{A}/\text{cm}^2$  asymptotically when  $C$  is decreased towards zero. It is interesting to note that for  $\eta \geq 0.5$  the threshold current is close

to its asymptotic value for  $C=0$ . For the "normal" case where  $\eta = 1$  the membrane capacitance can thus be changed between zero and about twice its actual value of  $1 \mu\text{F}/\text{cm}^2$  without affecting the threshold current much. This insensitivity to the value of  $C$  has been discussed by Colding-Jørgensen (1976).

Note that the threshold current  $i$  relative to its asymptotic value always increases as  $\eta$  is decreased, while the absolute value of  $i$  reaches a minimum value for  $\eta = 0.15$ . It was therefore found convenient to plot the firing frequency as a function

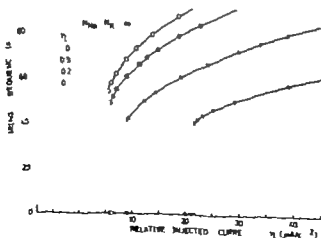


Fig. 2. Mean firing frequency as function of relative injected current  $\eta$  for different values of the relative maximal membrane conductance  $\gamma$  when  $\eta_0 = 1$ .

He also multiplied the specific membrane capacitance by a factor  $\gamma$  but this parameter will here be assumed constant equal to  $1 \mu\text{F}/\text{cm}^2$  in all nerve cells considered.

The stochastic calculations presented in the preceding paper were based upon the non-stochastic equations (1) and (7) in that paper (these are part of the H-H equations). With the above mentioned scaling these equations become

$$\frac{dE}{d\tau} = -\frac{\eta}{\phi C} (g_N (E - E_N) + g_K E + g_L (E - E_L) - i) \quad (1)$$

$$\frac{dx}{d\tau} = \alpha (1 - x) - \beta x$$

where  $x = h$  or  $n$  or  $\tau = t/\phi$  and  $i = i/\eta$ .

This set of equations is formally equivalent to Eqs (1) and (7) in the preceding paper where all conductances have been multiplied by the factor  $\eta = \eta/\phi$  since  $i$  is a free variable and can be chosen at will. In real time  $t$  everything will change  $1/\phi$  times slower than in these equivalent equations. These equations together with the total numbers  $N_N$  and  $N_K$  of sodium-conducting and potassium-conducting pores completely specify the stochastic equations. The numbers  $N_N$  and  $N_K$  are obtained from the pore densities and the membrane area of the space-clamped cell under consideration (or part thereof). In order to find the firing behaviour of the space-clamped membrane for all possible combinations of  $\eta$  and  $\phi$  it is thus sufficient to re-calculate the stochastic equations for different values of  $\eta = \eta/\phi$ . We may then arbitrarily assume  $\phi = 1$  in the calculations. In all the figures the frequency and current values shown are thus for  $\phi = 1$  and the value of  $\eta$  shown corresponds to the value of  $\eta$  in the general case where  $\phi \neq 1$ . In order to keep the discussion general we will therefore use  $\eta$  in the text even if we refer to figures where  $\eta$  is used.

Since the axon ceases to conduct the action potential when  $\eta \leq 0.05$  (Huxley 1959; Sabah & Leibovic 1977) the firing behaviour was investigated for  $\eta = 0.1, 0.2$  and  $0.5$ . The case  $\eta = 1$  has already been investigated in the preceding paper. In this special case where  $\eta = \eta/\phi = 1$  everything will change  $1/\phi$  times slower than shown by the figures in the preceding paper (the frequencies must for instance be multiplied by  $\phi$ ) and the scaled injected current  $i$  must be multiplied by  $\eta$  in order to obtain the real specific current.

But the peak amplitudes and the form of membrane potential changes will not be different.

The results obtained in this paper for a constant value of  $\eta = 1$  can also be treated in this way since the model time  $\tau = t/\phi$  is independent of  $\phi$ . The frequencies shown must thus be multiplied by  $\phi$ . Instead of the specific injected current  $i$  we found more useful to use the relative specific injected current  $i = i/\eta = i/\phi$ . In the figures the injected current is thus scaled according to the maximal ionic currents in the membrane because these are proportional to  $\eta$  and also scaled according to the rate constants because  $\phi$  is included in them.

In order to compare the results obtained with the non-stochastic H-H equations some calculations with these were made in order to find the steady state firing frequency as a function of injected current for different values of  $\eta$ . The results are shown in Figs 1 and 2. A method similar to the one described by Hodgkin & Huxley in their paper was used to solve the H-H equations (Hodgkin & Huxley 1952).

The methods used for solving the stochastic equations are described in the former paper (Skaugen & Walloe 1979). The mean firing frequency and an approximate value of the standard error  $\Delta f$  of the mean of this frequency were calculated as before (Skaugen & Walloe 1979). The value  $\Delta f$  is shown by vertical bars in the figures.

## RESULTS

Figs 1 and 2 show the results obtained by calculating the response of the original non-stochastic H-H equations to a step injected current for different values of the relative maximal conductances. These equations correspond to the stochastic equations used here but with an infinite number of sodium and potassium pores. A step injected current defined by  $i = 0$  for  $t < 0$  and  $i = 1$  for  $t \geq 0$  was chosen because the frequency-current relation then includes the lowest frequencies which can be sustained in a steady state firing. The threshold current for sustained firing is shown in Fig 1 as a function of  $\eta$ . For  $\eta$  smaller than about 0.08 it is difficult to decide whether in a given value of  $\eta$  we had sustained firing or not because the response was a train of action potentials with slowly decreasing amplitudes. It was necessary to calculate the response for an excessive long model time in order to decide whether the amplitudes decreased to zero (below threshold) or to a constant value larger than

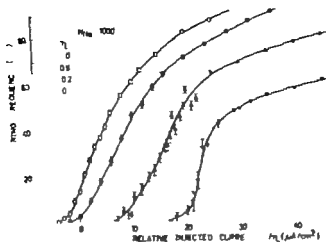


Fig. 5. Mean firing frequency as a function of relative injected current  $I/I_0$  for different values of the relative internal membrane conductance  $\eta$  when  $N_0 = 1000$  and  $N = \infty$ .

The firing frequency as a function of the relative injected current  $I/I_0$  is shown in Fig. 2 for different values of  $\eta$ . As  $\eta$  is decreased, the frequency-current curves are shifted to the right in the figure. This is due to the increase of the relative threshold current. It is noted that the steep (almost vertical) part of the curves at the threshold decreases somewhat in height as  $\eta$  is decreased, but the computations did in all cases indicate an equal abrupt increase of the frequency at the threshold.

Figs 3 to 6 show the mean firing frequency as a function of the relative injected current for dif-

ferent values of  $\eta$  and the pore numbers  $N_0$  and  $N$ . The curves for  $\eta = 1$  are also shown. These were calculated in the former paper (they are there shown in Figs 4 and 5) and are included here for completeness. As in the former paper the figures show the effects of a finite pore number in one of the two pore systems at a time. In Figs 3 and 4 the numbers  $N_0$  and  $N$  of sodium and potassium pores are  $N_0 = 100$  and  $N = \infty$  and  $N_0 = 100$ ,  $N = \infty$  respectively. In both cases the main effect of a reduction of  $\eta$  is to decrease the mean firing frequency  $f$  for a given value of  $I/I_0$ . The

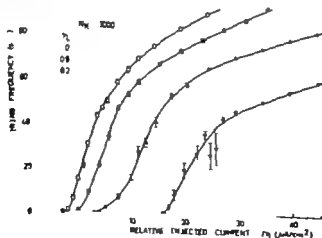


Fig. 6. Mean firing frequency as a function of relative injected current  $I/I_0$  for different values of the relative internal membrane conductance  $\eta$  when  $N_0 = 1000$  and  $N = \infty$ .



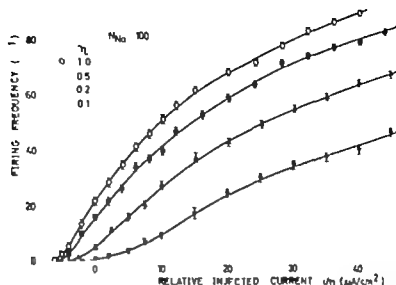


Fig. 3. Mean firing frequency as a function of relative injected current  $i/\eta$  for different values of the relative maximal membrane conductance  $\eta$  when  $\lambda_{Na} = 100$  and  $\lambda_{K} = \infty$ .

of the relative injected current  $i/\eta$  as this gave a systematic shift to the right of the frequency-current curves when  $\eta$  was decreased. If the parameter  $\phi$  is assumed constant this also means that the injected current is shown relative to the maximal ionic currents in the membrane.

The upper broken line in Fig. 1 given by  $i = \eta \cdot 154 \mu A/cm^2$  shows the upper limit to the injected current for sustained firing when the membrane capacitance is very small. This limit was found analytically by linearization of the H-H equations (Hodgkin & Huxley 1952). This is valid at this

limit because the amplitudes of the action potential then approach zero. The triangle bounded by the two broken lines thus shows the combinations of  $i/\eta$  and  $\eta$  where sustained firing is possible when  $C = 0$ . The continuous line shows the region when  $C = 1 \mu F/cm^2$ . If  $C$  is increased Eq. (1) is left unchanged if  $\eta$  and  $i$  are increased in proportion. This simple scaling makes it possible to find  $i/\eta$  as a function of  $\eta$  for any value of  $C$  from the curves for  $C = 1 \mu F/cm^2$ . Two examples for  $C = 0.5 \mu F/cm^2$  and for  $C = 2 \mu F/cm^2$  are shown by the dotted curves in Fig. 1.

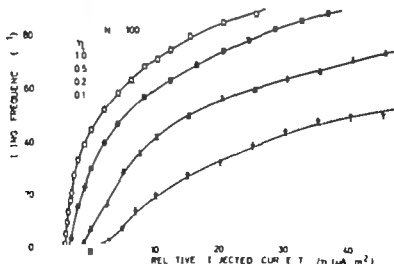


Fig. 4. Mean firing frequency as a function of relative injected current  $i/\eta$  for different values of the relative maximal membrane conductance  $\eta$  when  $\lambda_{Na} = 100$  and  $\lambda_{K} = \infty$ .



Fig. 8. Standard deviation of interspike intervals as a function of the mean firing frequency for different values of  $\eta$  and for  $N_{\text{ion}} = 1000$  (—) and for  $\eta = 1$  and  $N_{\text{ion}} = 20000$  (---).

frequency it was found that a larger value of the standard deviation  $\Delta T$  was connected with a greater degree of burst firing, either as seen directly on inspection of the spike trains, examples of which are given in Fig. 1, or as measured by the burst factor BF. (All these parameters are defined in the former paper.) I do not use the burst factor here because  $\Delta T$  changes in a more systematic way and is easier to interpolate.

The continuous lines in Fig. 8 show the standard deviation  $\Delta T$  as a function of the mean firing frequency  $f$  for different values of  $\eta$  and for  $N_{\text{ion}} = 1000$  and  $V$  infinite. For  $f$  smaller than about 40 s<sup>-1</sup>,  $\Delta T$  increases considerably when  $\eta$  is decreased. This supports the more qualitative results obtained on inspection of the spike trains, namely that a decrease in  $\eta$  is followed by an increase in the tendency to burst firing on the steep lower part of the frequency-current relationship. For firing frequencies  $f$  higher than about 40 s<sup>-1</sup>, the opposite is the case. But  $f$  is now too high to allow burst firing, a decrease in  $\Delta T$  here only means a more regular firing.

Note that this central frequency of  $f = 40$  s<sup>-1</sup> is roughly equal to the minimum frequency obtained or sustained firing when  $N_{\text{ion}}$  and  $N$  are infinite as shown by Fig. 1. This is in accordance with the

discussion in the former paper of the mechanisms underlying burst firing.

The broken line in Fig. 8 shows  $\Delta T$  as a function of  $f$  for  $\eta = 1$ ,  $N_{\text{ion}} = 20000$  and  $N$  infinite. This curve is close to the curve for  $\eta = 0.1$  when  $N_{\text{ion}} = 1000$  and  $f \sim 20$  s<sup>-1</sup>, which indicates that a reduction of  $\eta$  from 1 to 0.1 gives approximately the same increase in burst firing as an increase of the pore number from 1000 to 20000. Corresponding results were obtained for  $V = 1000$  and  $N_{\text{ion}}$  infinite.

For smaller pore numbers  $N_{\text{ion}} = 100$  or  $N = 100$ , there was a small systematic change in  $\Delta T$  when  $\eta$  was reduced, but this change could not be considered significant. There was thus no significant increase in the burst firing when  $\eta$  was decreased at these pore numbers. At the same time we note that an increase of the pore number from 100 to 1000 does not significantly increase the tendency to burst firing when  $\eta = 1$ , either. This is seen from Figs. 8 and 9 in the former paper.

The general conclusion from these results is that assuming  $\phi$  constant, a reduction of the pore densities does not alter the firing behaviour drastically when the pore numbers are kept constant. The frequency-current relationship may change somewhat, depending on the number of pores. In addition the tendency to burst firing is increased when the pore densities are reduced.

Decreasing the pore densities while keeping the pore numbers constant means increasing the area of the nerve membrane which are simulated. Another way of looking at these results would be to assume the area constant. A reduction in the pore densities then gives a corresponding decrease in the pore numbers. In this case a reduction of the pore densities will not change the tendency to burst firing much according to the discussion above, at least not within the range 100 to 10000 pores. If the relative injected current is used, the frequency-current relationship will become less steep and more linear and it will be shifted somewhat towards larger currents. This is illustrated in Fig. 9 where the relative pore density  $\eta$  and the sodium pore number  $N_{\text{ion}}$  are reduced from  $\eta = 1.0$  and  $N_{\text{ion}} = 3000$  to  $\eta = 0.1$  and  $N_{\text{ion}} = 1000$  in the upper part A of the figure and from  $\eta = 1.0$  and  $N_{\text{ion}} = 1000$  to  $\eta = 0.1$  and  $N_{\text{ion}} = 100$  in the lower part B.

## DISCUSSION

The effects upon the firing behaviour of a finite number of pores are likely to be greatest in a small

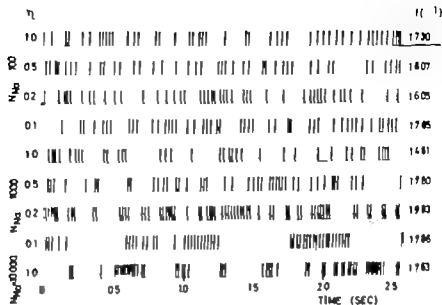


Fig. 7. Examples of trains of action potentials obtained for different values of the relative maximal conductances  $\eta$  and the number  $N_{Na}$  of Na-pores.  $N_K = \infty$ .

frequency-current relationship becomes less steep (using  $1/\eta$ ) and if anything more linear. It also takes on a more distinct sigmoid form. It should also be noted that while the curves with  $\eta = 1$  are rather different in the two cases shown by Figs. 3 and 4, the curves become more similar in shape as  $\eta$  is reduced. The standard error of the mean shown by the vertical bars seems to be more or less the same for values of the different parameters.

For larger pore numbers the case is somewhat different as shown by Figs. 5 and 6, where  $N_{Na} = 1000$  and  $N_K$  is infinite and  $N_K = 1000$  and  $N_{Na}$  is infinite respectively. The shape of the frequency-current relationship seems to be more or less independent of  $\eta$ . The steepness of the curves changes very little, the only exception is for  $\eta = 0.1$  when  $N_K = 1000$  and  $N_{Na}$  is infinite, but the frequency decreases somewhat when  $\eta$  decreases. This latter phenomenon is rather similar to the changes noted in the curves for infinite values of both  $N_{Na}$  and  $N_K$  shown in Fig. 7. The shift of the curves to the right when  $\eta$  is decreased is also seen, but it is somewhat different from that shown in Fig. 7. For these larger pore numbers ( $N_{Na} = 1000$  and  $N_K = 1000$ ) the curves for the same values of  $\eta$  are almost similar for the two pore systems considered, sodium in Fig. 5 and potassium in Fig. 6.

For the smallest values of  $\eta$  shown there is also a marked difference in the magnitudes of the stand-

ard error of the mean of  $f$  as shown by the vertical bars. On the steep lower part of the curve they are larger than for larger values of  $\eta$ , while for the upper part of the curve they are smaller. This is due to a pronounced burst firing on the steep lower part and a quite regular firing on the upper part of the curve. This is similar to the firing behavior for even larger pore numbers, 5000–10000 pores, and above when  $\eta = 1$ , as shown in the first paper. This is partly illustrated in Fig. 7, where typical trains of action potentials obtained in simulation are shown in the case where  $N_K$  is infinite and for different values of  $N_{Na}$  and  $\eta$ . The mean firing frequency is in all cases about 17 Hz, that is, on the steep lower part of the frequency-current relationship. For  $N_{Na} = 100$  there are marked differences to be seen in the spike frequency for different values of  $\eta$ . But when  $N_{Na} = 1000$ , an increase in burst firing is seen when  $\eta$  is reduced from 0.7 to 0.1. At the bottom of the figure a 4-train for  $N_{Na} = 10000$  and  $\eta = 1$  is shown. The tendency to burst firing is also here quite clear, perhaps more similar to the case where  $\eta = 0.1$ ,  $N_{Na} = 1000$  than to the other cases shown.

The standard deviation obtained in the simulation can be used to indicate the degree of burst firing. We then use the standard deviation  $\Delta T$  of the interspike intervals, which is more precisely defined than the standard deviation of the mean firing frequency shown in the figures, for a given mean

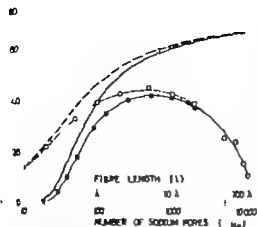


Fig. 10. Mean firing frequency in real nerve fibre as function of fibre length in different cases. 100 pores over one space constant. See text for explanation.

If the action potential is constant when measured with the scaled time  $\tau = \phi / \lambda$  which gives  $t \sim \lambda / \phi$  which gives a change in the  $t / \phi$  of only 25% over the observed velocity range 6 m/s to 3.0 m/s. The product  $t / \phi$  will be closer a constant if  $\phi$  decreases faster than  $\lambda$  that is,  $\lambda \sim \phi^2$  increases when  $d$  decreases.

The parameters observed,  $\phi$  and  $\lambda$  are not very sensitive to  $\mu$  and  $\eta$ . If we for instance assume  $\eta = 10^{-4} - 10^{-5}$  we obtain from Huxley's dimensional analysis  $\phi \sim d^{0.5}$  and  $\lambda \sim \phi^{-0.5}$  against  $d^{0.5}$  and  $\phi^{-0.5}$  for  $\phi = \eta$ . As  $d = 0.2 \mu\text{m}$  this gives  $\eta = 15$  which gives a peak amplitude of 75% of that found at  $\eta = 1$ . This latter result is in agreement with observations which suggest that the peak amplitude is smaller in small fibres than in large ones.

We see that the scaled H-H equations give results that agree qualitatively with the experiments done in small unmyelinated nerve fibres and that the results obtained here may indicate the effects upon the firing behaviour of small nerve fibres due to a finite number of pores. However it is necessary to establish some connection between the space-clamped, insulated membrane studied here and the physiological operating nerve cell in order to be able to say anything more definite about this. This has been done below. The conclusion is, it is sufficient to find the number of pores over a few space constants.

Let us assume that we are studying the middle

part of a very long uniform unmyelinated nerve fibre where the whole fibre is uniformly stimulated by a specific injected current  $I$ . (The current can be envisaged as being applied through an axial electrode running along the whole length of the fibre.) In case A a piece with length  $l$  of this fibre is insulated at both ends, and the internal resistance of this piece is zero. This is equivalent to the case studied in these papers. The pore numbers are proportional to the length  $l$  of the fibre as an example in Fig. 10 one hundred sodium pores are assumed to cover one space constant  $\lambda$ . The mean firing frequency as a function of  $l$  will then be as shown by the thin broken line in Fig. 10, which is taken from Fig. 10 in Skaggen & Walløe (1979). The  $\lambda$  does signify the space constant that this fibre model would have had in the presence of a normal non-zero resistance. The specific injected current  $I$  is in all cases shown in this figure equal to  $5 \mu\text{A}/\text{cm}^2$  smaller than the firing threshold in the H-H equations.

The thin, continuous line shows case B where a length  $l$  of the same nerve fibre is loaded at both ends with the input resistance of the real nerve fibre but where the internal resistance of the piece studied still is assumed zero. The input resistance  $R_{in}$  is given by  $R_{in} = R \lambda / \pi r^2 = (2r G_m \lambda)^{-1}$  where  $R$ ,  $G_m$  and  $\lambda$  are the internal specific resistance, the membrane specific conductance the fibre radius, and the space constant  $\lambda = \sqrt{r / (\pi G R)}$  respectively. This case corresponds approximately to case A, but where the effective injected current is  $I_{eff} = I - 2(E_T - E_0) / (AR_{in}) = I - 2\lambda / (1 - I/I_T)$  where  $E_T$  is the threshold potential,  $E_0$  is the potential with the injected current  $I$ ,  $A = 2\pi r l$  is the area of the membrane and  $I$  and  $I_T$  are the specific injected and threshold currents respectively. Figs. 3 and 4 in the former paper were used to find this curve.

The heavy continuous curve shows case C which corresponds to case B but where the internal resistance is taken into account. This is a situation not far from a physiologically operating nerve cell or fibre. This curve cannot be calculated by the methods used here but its form can be indicated qualitatively from a number of its properties.

(a) It must approach case B when  $l$  is decreased towards zero ( $l \ll \lambda$ ) because the internal resistance then approaches zero.

(b) For the same length  $l$  the mean firing frequency in case C must always be higher than in case B because the internal resistance both makes

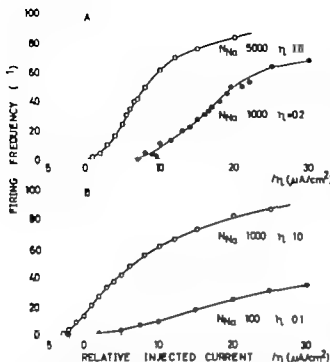


Fig. 9. Mean firing frequency as a function of relative injected current  $I/\eta$  for different values of  $\eta$  and  $N_{Na}$ . Upper part A for  $N_{Na} = 5000$   $\eta$  and lower part B for  $N_{Na} = 1000$   $\eta$ .  $\circ$   $\eta = 1$ ,  $\bullet$   $\eta = 0.2$  (A) and  $\eta = 0.1$  (B).

nerve cell where the total number of pores is much smaller than in a larger nerve cell. For the largest nerve cells or fibres such as the squid giant axon we would not expect any effects at all. It would thus be of great interest to know if the H-H equations can be used to describe approximately the firing behaviour in smaller nerve cells because the results obtained here and in the former paper could then be used at least qualitatively for these nerve membranes also. Unfortunately direct measurements of the membrane properties are at present only available for a few large axons but some measurements have been reported mainly of conduction speed  $\Theta$  but also of rise time for unmyelinated nerve fibres (Jack 1975). It is possible to indicate that these measurements can at least be quantitatively explained by equations of the same form as the H-H equations but with different rate constants and maximal membrane conductances as given by Eq. (1).

In order to make discussion simpler we will assume that the pore density  $\rho$  is proportional to  $\eta$ . This gives single pore conductances which are independent of the pore densities. But this is not necessary in order to use the results presented here because the equations are independent of the

actual pore densities or the single pore conductances. In any case it is not likely that single pore conductances differ very much between different nerve cells as it is believed that the size of pore is an important parameter in its ionic selectivity. The observed range of sodium pore density from 500 pores/ $\mu m^2$  in the 700  $\mu m$  thick nerve in squid to 2.5 pores/ $\mu m^2$  in the 0.2  $\mu m$  thin garfish thus also indicates a value of  $\eta$  of 0.06, the latter taking  $\eta = 1$  in squid (Keynes & 1974 Colquhoun et al. 1972). This small value of  $\eta$  is far below the value  $\eta = 0.05$  where impulse conduction fails if no other parameters are changed.

J. J. B. Jack has discussed the probable change in (specific) membrane parameters in nerve fibres of different sizes and he argues that the rate constants must be smaller in small nerve cells than large ones (Jack 1975). From Eq. (1) it is seen that a decrease of  $\eta$  is counterbalanced by a corresponding decrease of  $\phi$ . A small value of  $\phi$  would thus make possible a small value of  $\eta$  without stopping impulse propagation. A small value of  $\phi$  also accounts for the slow rise of the action potential found in small nerve fibres. The relative rate constant  $\phi$  is probably the only single parameter which can be changed sufficiently to account for the observations.

As a quantitative example we can assume that membrane parameters are power functions of the fibre diameter  $d$ . This follows the presentation in Jack's article from which the data used here are taken (Jack 1975). Using the observed extremes in fibre diameter  $d$  and pore density  $\rho = \rho_{Na}$  in squid and garfish we find  $\eta \sim \rho_{Na}^{-0.5}$  ( $\eta = (d/700 \mu m)^{-0.5}$ ). For conduction to take place we must have  $\eta \phi > 0.05$  at  $d = 0.2 \mu m$  which gives  $\phi < \eta^{-0.5}$ . Assuming  $\phi$  to be a certain power of  $d$  now determines all the non-stochastic properties of the fibre. For instance the conduction speed will be proportional to  $\sqrt{d}$  due to purely geometrical scaling and function of  $\eta$  and  $\phi$  as found in the "dimensional analysis" developed by Huxley (1959). This is scaling of the H-H equations similar to that shown in Eq. (1) but in this case of a propagated action potential. In the special case  $\phi = \eta$  it gives  $\Theta$  proportional to  $\sqrt{d}$  and then we have  $\Theta \sim \sqrt{d} \sqrt{\eta} \sim (d^{0.5} \eta^{0.5}) = d^{0.25}$ . This agrees reasonably well with experiments where it is found that  $\Theta \sim d^{0.25}$  in the unmyelinated fibre. An approximate inverse relation between rise time  $\tau$  and  $\Theta$  has also been measured. In our case in the time  $\tau$

guboun et al 1972). A specific internal resist-  
 $c R = 100 \Omega\text{-cm}$  and a membrane conductance  
 and the threshold of  $G_M = (p_M/300 \text{ pores}/\mu\text{m}^2)$   
 $m^2 S/cm^2$  is assumed. The conductance of  
 a pore is assumed constant and the scaling  $\phi = \eta$   
 used.

For the smallest nerve fibres the stochastic fluc-  
 tuations of the membrane potential due to a finite  
 number of pores should significantly influence the  
 firing behaviour. The frequency-current relation-  
 ship is for instance distinctly more linear for pore  
 fibres up to 20 000  $\mu\text{m}$  which is much higher than the  
 smallest numbers found in this table.

Recently a separate gating current has been  
 found in the nerve membrane in addition to the  
 sodium currents (Keynes & Rojas 1974; Armstrong  
 & Bernstein 1974). This current is not explicitly  
 included in the H-H equations and its inclusion  
 would be expected to change the firing behaviour  
 somewhat. But since the H-H equations are an  
 empirical fit to the observed, voltage-clamped be-  
 haviour, the main effects of the gating current must  
 already be included although not as a separate  
 mechanism. If the gating current is included as a  
 separate mechanism, the parameters of the original  
 H equations must be changed somewhat in order  
 to remove the effects of the gating current from  
 the model. In one instance the membrane capacitance  $C$   
 must for instance be adjusted down from  $1 \mu\text{F}/\text{cm}^2$  to  
 $0.88 \mu\text{F}/\text{cm}^2$  (Adrian 1975).

The effects of gating currents connected with the  
 variation of the sodium conductance can be ad-  
 justed explicitly in Eq. (1) by adding a term  $C$   
 $d\phi/dt$  where  $Q$  is the total charge associated with  
 the gates per unit area of the membrane. If the  
 conductances are multiplied by  $\eta$  and the conduct-  
 ance of each pore is assumed constant, the sodium  
 pore density and hence  $Q$  is also changed by a  
 factor  $\eta$ . At the same time  $t$  is replaced by  $\eta t$  and  
 $d\phi/dt$  must be replaced by  $\eta Q d\phi/dt$  ( $\eta$  is then  
 outside the parenthesis).

$$\frac{d\phi}{dt} = \frac{\eta}{C} \left( (x_{Na}(E - E_{Na}) + \phi Q) \frac{d\phi}{dt} + R_p E + \right) \quad (2)$$

$$(E - E_{Na})$$

This shows that the relative effects of the gating  
 current are proportional to  $\phi$ , and thus propor-  
 tional to the pore density if the scaling  $\phi = \eta$  is used.  
 In any case this effect will be very slight in small  
 nerve cells or nerve fibres. It would then perhaps be

more correct to use the adjusted value  $0.88 \mu\text{F}/\text{cm}^2$   
 for the specific membrane capacitance  $C$  in our  
 equations (Adrian 1975). But it would not change  
 the result much as it merely corresponds to a slight  
 by larger value of  $\eta$ .

The latest experiments seem to confirm the  
 existence of a two-state sodium system (Conti et al  
 1976). But a multistate pore system is still a possi-  
 bility especially for the potassium pore system  
 and at any rate in other types of nerve cells. In a  
 three-state pore system, for instance, each pore  
 could be shut, half open or fully open. This cor-  
 responds roughly to the two-state pore system but  
 with twice the number of pores. In general how-  
 ever the states in one three-state pore would be  
 coupled and thus it could not be represented by  
 two independent two-state pores. In this case  
 separate computations would be needed to find the  
 firing behaviour.

The potassium pore system is not so well investi-  
 gated experimentally as the sodium pore system,  
 and it may well be that the potassium pores do not  
 operate on an on-off basis, even if it is fairly certain  
 that the sodium pores do. The effects upon firing  
 behaviour due to a finite number of potassium pores  
 will then be less than shown here. Hille estimates  
 the potassium pore density to be fifty times larger  
 than the sodium pore density (Hille 1970) and in  
 that case our model predicts a firing behaviour very  
 close to that obtained by assuming an infinite num-  
 ber of potassium pores. On the other hand Conti et al  
 argue that the conductance of a single open  
 potassium pore is three times larger than that of a  
 single open sodium pore (Conti et al 1975). This  
 gives a potassium pore density ten times smaller  
 than the sodium pore density and the firing be-  
 haviour will be dominated by the potassium pore  
 system if its pores operate on an on-off basis.

There are several sources of electrical noise in  
 the nerve membrane in addition to the stochastic  
 fluctuations of the membrane conductances con-  
 sidered here. One example is the thermal noise.  
 These other types of membrane potential fluctua-  
 tions will give effects which come in addition to the  
 effects shown here. The results presented here thus  
 show lower limits to the expected electrical fluctua-  
 tions in more realistic models where all membrane  
 sources of noise have been included. According to  
 Lecar and Nossal's discussion however it seems  
 that the potential fluctuations in the node of Ranvier  
 are mainly due to sodium conductance fluctuations.

Table 1 Number of sodium pores over two space constants in nerve fibres of different types calculated according to

$$N_{Na} = \rho_{Na} \pi d (d / (G_m R_i))^{1/2} = (\rho_{Na} d^3)^{1/2} \cdot 6680 \left( \frac{\text{pores}}{\mu\text{m}} \right)^{1/2}$$

Type of fibre	Diameter $d$ ( $\mu\text{m}$ )	Sodium pores per $\mu\text{m}^2$	Area over two space constants $A$ ( $\mu\text{m}^2$ )	Number $N_{Na}$ of sodium pores over two space constants
Squid giant axon [7]	500-900	500	$3.4 \cdot 10^4 - 1 \cdot 10^5$	$1.7 \cdot 10^4 - 1 \cdot 10^5$
Rabbit vagus nerve [8]	0.3-1.95	77	$1 - 3510$	$570 - 96000$
Lobster leg nerve [8]	0.2-30.0	16	$150 - 27500$	$400 - 4 \cdot 10^4$
Garfish olfactory nerve [8]	$\sim 0.2$	2.5	379	948

the current lost through the load  $R_i$  at both ends smaller and makes the local potential fluctuations in any part of the fibre larger. Due to the internal resistance the local fluctuations at any given place in the fibre will be at least slightly different from fluctuations at other places and they will be larger than the fluctuations of the space mean of the potential.

(c) The mean firing frequency  $f$  is a monotonically increasing function of  $I$ . If the nerve fibre of length  $l$  is divided in two pieces of lengths  $l_1$  and  $l_2$  each of the pieces will be loaded at both ends with the fibre input resistance  $R_i$  and thus be approximately equal to two separate pieces. The combined firing of the two pieces will be higher than the firing in anyone of them but in general  $f$  will not be a linear function of  $I$  because the two pieces will affect each other's fluctuations somewhat. When  $f$  is increased an increasingly larger part of the time will be spent with one of the pieces in a refractory state due to an action potential generated in the other piece. When  $l$  is increased  $f$  will therefore approach a certain limit which in any case cannot be larger than the maximum firing rate of the fibre.

Case D is shown qualitatively by the heavy broken curve. It corresponds to case A where the fibre is insulated at both ends but with the internal resistance taken into account. At any value of  $I$  the mean firing frequency  $f$  will be larger in case D than in case A because the local potential fluctuations in any part of the fibre will be larger and larger than in case C because there is no current lost through the ends of the fibre. As  $l$  increases and approaches infinity ( $l \gg \lambda$ ) cases C and D must approach each other and become practically identical because the current lost through the ends in case C will

become insignificant compared to the current lost through the membrane.

In all cases the space constant  $\lambda$  is a very important parameter. At  $l = \lambda$  the input resistance  $R_i$  equal to the membrane resistance  $1/g_m = (G_m)^{-1}$  and local fluctuations of the membrane potential begin to become important because the internal resistance  $R_i/(\pi r^2)$  then also is equal to  $1/g_m$ . The mean firing frequency  $f$  is equal in cases A and B when the fibre length  $l$  is in the range  $l \sim \lambda$  (Fig. 10) to a few times  $\lambda$ . This relation depends upon the injected current  $I$  and the combination of pore numbers  $N_{Na}$  and  $N_K$  used.

In a physiologically operating nerve cell the geometry and dimensions around the site for spike generation determines the effects of finite number of pores upon the firing behaviour. In many cases this site is part of or connected to the axon and can be represented by a long thin fibre. A lot of a few space constants of this fibre will according to the discussion above have roughly the same firing behaviour as the membrane model studied here when the pore numbers are the same. We can thus get an idea of the firing behaviour of nerves by finding the numbers of pores over a few space constants in the axon around the site of spike generation if known and then using the figures presented here. In general both the mean firing frequency  $f$  and the specific injected current  $I$  are scaled. The real values of  $f$  and  $I$  are found by multiplying the  $f$  and  $I$  shown in the figures by  $\phi$  and  $\eta = \eta/\phi$  respectively.

Table 1 shows examples of the total number of sodium pores over two space constants for nerve fibres of different sizes where the sodium pore density has been measured (Keynes & Rojas 1970).

# the postnatal development of the inferior oblique muscle in the cat

## Fiber sizes and histochemical properties

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HANSON E, LENNERSTRAND G & NICHOLS K. C. The postnatal development of the inferior oblique muscle of the cat. III. Fiber sizes and histochemical properties. *Acta Physiol Scand* 1980; 108: 61-71. Received 8 May 1979. ISSN 0001-6772. Departments of Clinical Neurophysiology and Neurology, Karolinska Hospital, Stockholm and Department of Ophthalmology, University Hospital, Linköping, Sweden.

Transversal sections of the inferior oblique muscle from litters of various ages and from adult cats were stained for myofibrillar ATPase at pH 9.4 and 4.35, succinic dehydrogenase (SDH) and fat. The same muscles had previously been submitted to studies of contractional and fatigue properties. With ATPase the fibers could be differentiated into types I, II and IIC. The percentages of each fiber type remained approximately the same from birth onwards. In the inner global layer of the muscle, type I fibers showed a linear increase in size, but types II and IIC fibers an accelerated growth after age 70 days. In the outer orbital layer, there were no type I fibers were seen, types II and IIC fibers showed the same growth pattern as in the global layer. The fiber content of SDH and fat was low at birth but increased after two weeks of age. Type I fibers were poor and type II fibers rich in SDH and fat. Type II fibers showed varying amounts of these substances. Provided that type I fibers are slow, type II fast and type IIC intermediate in speed of contraction, like in other muscles, the findings on fiber growth and SDH content seem to support the idea that slow, fatigue-resistant components in eye muscles reach maturity earlier than fast components.

*Key words:* Postnatal development, cat extrinsic muscle, fast and slow fibers, histochemistry.

It has been suggested from physiological data on the prenatal development of the cat inferior oblique muscle that slowly contracting fibers reach maturity later than rapidly contracting fibers (Lennérstrand & Hanson 1978, b). It was thought that histochemical study of developing eye muscles would further elucidate the time course of the differentiation of fast and slow fibers. Earlier work on motor unit in hindlimb muscle has shown a close relation between the histochemical staining pattern of the fibers and physiological properties like conduction velocity and resistance to fatigue (Lennérstrand & Kugelberg 1968, Burke et al. 1973, Kugelberg 1973). It seems very likely that this relation holds also for developing hindlimb muscle of the cat (Nygren 1968, Hammarberg 1975). It

has been confirmed in adult rat and cat eye muscles (Hanson & Lennérstrand 1977) but has not yet been studied in eye muscle of the kitten.

In this investigation the same muscles were examined with histochemical techniques that had been used for the physiological experiment on eye muscle development. Preliminary reports have been published elsewhere (Lennérstrand, Hanson & Nichols 1977, Hanson, Lennérstrand & Nichols 1977).

## METHODS

### Histochemical techniques

The physiological experiments on the inferior oblique muscles (IO) of 25 kittens and 3 adult cats were described in previous papers (Lennérstrand & Hanson 1978a, b).



(Lecar & Nossal 1971). They did not consider the potassium conductance. In the membrane model presented here the potassium conductance fluctuations were also found to be important, but this may be at least partly due to the relatively large importance of the potassium current in the squid axon as compared with the myelinated nerve.

In addition to the noise due to membrane processes, there is also electrical noise in the membrane potential due to synaptic activity. Both the quantal release of transmitter substance and the irregular arrival of nervous impulses from other nerve cells or irregular stimulation from sense receptors will be sources of this "noise". This variability in the excitation will in many cases probably be a far more important source of membrane potential fluctuations than the membrane processes themselves, especially in large nerve cells, but is not considered here. The injected current is assumed to be free of any random fluctuations.

The space-clamped nerve membrane was chosen as a basis for the model used here in order to simplify the calculations, and to be able to compare the results with those of a well-known model. But it is of course of more general interest to find how the firing behaviour of a nerve cell in a physiological situation is influenced by a finite number of pores. One must then solve the set of equations chosen with both time and space as variables. The geometry of the nerve membrane which defines the surface of the cell is then important. This has been done for some simple cases, and the result will be presented in a later paper.

I would like to thank David Attwell and Lars Walløe for stimulating discussions, Daniel Kernell and Arild Njå for comments on the manuscript, and Torhild Isachsen for drawing the figures. Part of the work was done at the University Laboratory of Physiology, Oxford. It is a pleasure to thank Julian J. B. Jack and David Whettnidge for their kind hospitality and for providing the computer time needed. The work was supported by The Norwegian Research Council for Science and the Humanities.

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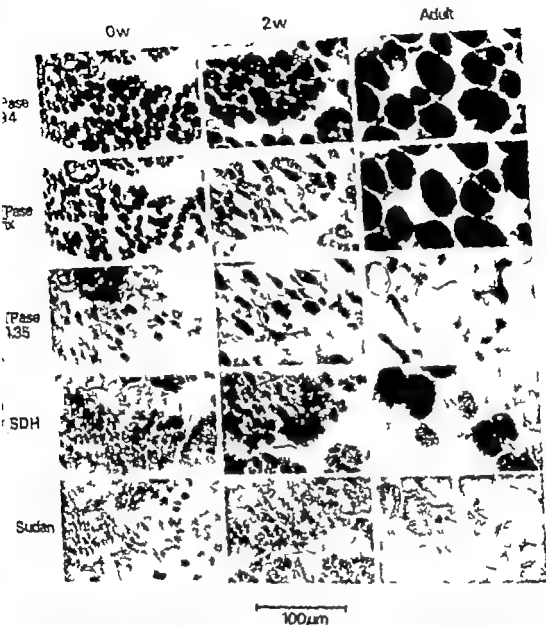


Fig. 1. Microphotographs of areas from the global layer in animals of different ages. Connective sections stained in Sudan. Note the clear differentiation in fiber types with ATPase stainings already in the newborn kitten. 1. SDH and 2. Sudan. The differentiation became clearer with age.

In the adult animal type I fibers were lightly to moderately stained and orbital type II as well as subal and orbital type IIC fibers heavily stained. Subal type II fibers varied from light to dark in SDH staining intensity. The changes in Sudan staining with age and fiber types seemed to parallel those in ATP activity but with less marked variations.

In a fascicle of the very young muscles stained for ATPase 9.4 the dark fibers were often seen in a row around the slightly larger light fibers like the petals of a flower (Fig. 3). Davies (1977) has described a similar arrangement in porcine muscle. It could occasionally be seen also in muscles from older kittens and adult animals but the arrangement be-

When tension recordings were completed the muscles were removed and frozen in Freon cooled to  $-160^{\circ}\text{C}$  with liquid nitrogen. To facilitate handling, very small muscles were adhered to a piece of meat prior to freezing. Muscles were stored at  $-85^{\circ}\text{C}$  for periods between one week and three months. Serial cross-sections ( $10\ \mu\text{m}$ ) cut in a cryostat were taken from the middle third of the muscle, close to the nerve entrance.

Sections were stained for: (1) ATPase after pre-incubation at pH 9.4 (ATPase-9.4) (Padykula & Herman 1955, revised by Brooke & Kaiser 1970); (2) ATPase after pre-incubation at pH 4.35 (ATPase-4.35) (Brooke & Kaiser 1970); (3) ATPase after postfixation with formaldehyde (ATPase-fix) (Hayashi & Freeman 1966); (4) Succinate dehydrogenase (SDH) (Nachlas et al. 1957; Pearce 1960); and (5) Lipids (Sudan Black) (Carleton & Drury 1957).

#### Measurements performed

The relative area occupied by the global (including when visible the global central) and the orbital layers were estimated (see Alvarado & van Horn 1975). Measurements were made with a planimeter on microphotograph ( $113\times$ ) of the sections stained for ATPase-fix. Area representative of the different layers in each muscle were photographed in all stains. Photographs ( $830\times$ ) of samples from the formaldehyde and acid preincubations were printed for determination of stain intensity and fiber size. In each sample a random line was drawn across the photograph and 40 consecutive fibers touching this line were graded for stain intensity. The two longest fiber diameters at right angles were used for area calculations. Cells having one diameter two or more times the other were rejected as not being in cross-section. The largest fibers had a greater chance of hitting the line. The probability of a fiber touching the line was considered roughly proportional to the sum of the two measured diameters of the fibers. The observed percentages and area were corrected accordingly. The cross-section area of each fiber was calculated assuming it to be ellipsoidal. Usually the 10 muscles from both eyes were examined. Differences between the eyes were assumed to be random.

For ATPase-fix and ATPase-4.35 sections average values were calculated in each age group for: (1) percentage of fibers which stained darkly and lightly and (2) average cell area of each of the fiber types. Analysis of variance (one way) was used for determining difference between age groups. When necessary for a more detailed comparison between age groups, confidence intervals were calculated according to the method of Schaffé (1961). In order to determine histochemical profile of the fibers, comparisons of the same 15 to 20 fibers in all stains were made by direct observation through the microscope.

## RESULTS

### Layering of the muscles

In the adult animal the IO muscle is divided into two main parts: the global layer with large fibers located on the inner global side of the muscle and the orbital layer with small fibers located on the

outer orbital side (Mayr 1971; Alvarado & Horn 1975; Hanson & Lennernstrand 1977). The layers were scarcely discernible at birth and not until 10 weeks of age that the layering was distinct as in adult muscles.

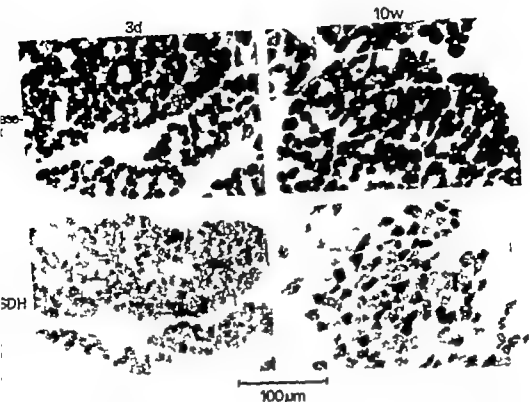
In animals 2 weeks of age and older, some bundles seemed to belong to a third global layer between the two main layers. The fibers in these bundles were most similar to those of the global layer, although they were slightly smaller and often arranged more compactly in the bundles. They have been included in the global layer in calculating the cross section areas of the different muscle parts.

The estimated area of the orbital layer differed from about 20% of the entire transverse area of the muscles in the young animals to about 10% of the total muscle area in adult animals. At very early age, and therefore values obtained in very young animals were less exact than those from older animals.

### General histochemical features and fiber types

At all ages, a high proportion of the fibers showed marked activity of ATPase after incubation at pH 9.4 (with or without postfixation with formaldehyde). Most of the fibers that were dark after incubation were left unstained after treatment at pH 4, which indicates that they were of type II (Brooke & Kaiser 1970). Such fibers were seen in both global and the orbital layers (Figs 1 and 2). The pale fibers in ATPase-9.4 reversed their color in ATPase-4.35 and became rather dark. The majority of type I fibers according to Brooke & Kaiser (1970). These fibers existed only in the global layer (Fig. 1). In addition to these two fiber types, a few fibers were seen. About 1/5 of the fibers in the orbital layer and 1/10 of the fibers in the global layer stained darkly for ATPase both at pH 9.4 and 4.35. They must be considered type IIC fibers (Brooke & Kaiser 1970).

There was much less differentiation between fiber types in SDH activity at birth than in ATPase. Type I fibers were pale in SDH with a few large fibers with intense color. Type II fibers were dark. Further differentiation appeared in type II SDH activity began at about 4 weeks. The SDH activity of the muscle seemed to increase between 2 and 6 weeks of age but the characteristic differences in SDH activity between types I and II fibers remained the same throughout development.



Sections from the global layer stained for ATPase- $\text{fix}$  (kittens aged 3 days and 10 weeks respectively). The flower arrangement of the fibers in ATPase- $\text{fix}$  with dark fibers surrounding the fibers in most marked in the postnatal sections. Here the light fibers are the largest.

types I, II and IIC fibers of the global layer. Types II and IIC fibers of the orbital layer. In SDH activity was included in the differentiation. The type II global fibers could be subdivided into fibers with high and low activity. For the other types SDH activity was the same within each type. The significance of this histochemical fiber presentation for the ultrastructural separation of fiber types in adult cat eye muscle (Alvarado & Smith 1975) will be discussed later. The relative portions of each fiber type determined in consecutive sections from the same muscles are given in Table 1.

#### Adult eye fiber type and size changes

Adult proportions of fibers with dark and light color ATPase- $\text{fix}$  remained fairly constant during postnatal development (inset of Figs 4 and 5). This was confirmed with an analysis of variance. Size of the type II fibers (dark in ATPase- $\text{fix}$ , light in ATPase- $\text{fix}$ ) increased steadily up to

20 weeks of age, followed by an accelerated increase between age 20 weeks and the adult stage. Type I fibers, which were light in ATPase- $\text{fix}$  and dark in ATPase- $\text{fix}$ , increased linearly through the whole age span. Type IIC fibers, dark in both ATPase- $\text{fix}$  and - $\text{fix}$ , were not identified and measured separately, since it was impossible to trace fibers; both stains only in a few muscles of each age group. The general impression is that IIC fibers were smaller than types I and II fibers, and similar in growth pattern to the type IIC fibers in the orbital layer. The proportion of the global type IIC fiber remained the same throughout development.

In the adult cat the type II fiber mean area was significantly larger than type I fiber area ( $P < 0.05$ ). At ages below 20 weeks there was no significant difference between the groups.

#### Orbital layer fiber types and size changes

All fibers of the orbital layer stained darkly in ATPase- $\text{fix}$  in muscles of all ages (Fig. 6 inset).

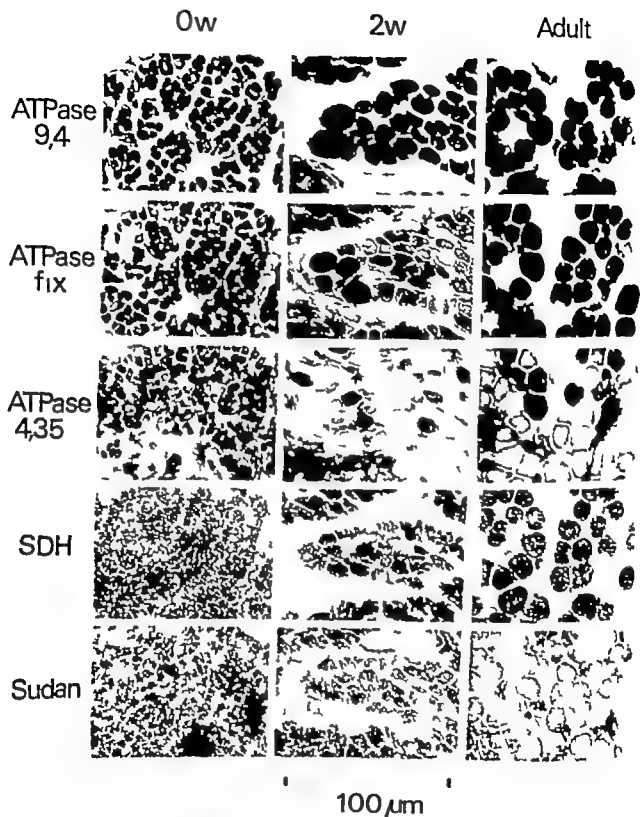


Fig. 2. Microphotographs of consecutive sections from orbital areas of the same muscle as in Fig. 1. All cells are dark in ATPase 9,4. ATPase-fix and SDH. In ATPase-4,35 type II and IIC fibers can be differentiated already at birth.

came less clear with increasing age as the darker eye muscle fiber types in the ATPase stainings. The distribution of the fibers in the two layers of the muscle was also taken into account. These type

In the adult cat it was possible to distinguish five

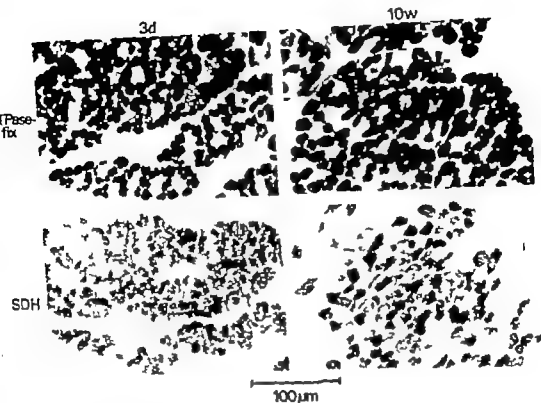


Fig. 3. Sections from the global layer stained for ATPase-fix (kittens aged 3 days and 10 weeks respectively). The flower-like arrangement of the fibers in ATPase-fix with dark fibers surrounding the fibers, is most marked in the youngest kitten where the light fibers are the largest.

are types I, II and IIC fibers of the global layer and types II and IIC fibers of the orbital layer. When SDH activity was included in the differentiation, the type II global fibers could be subdivided into fibers with high and low activity. For the other fiber types SDH activity was the same within each group. The significance of this histochemical fiber differentiation for the ultrastructural separation of fiber types in adult cat eye muscle (Alvarado & van der Horst 1974) will be discussed later. The relative proportions of each fiber type determined in consecutive sections from the same muscles are given in Table 1.

#### Adult cat fiber proportions and size changes

The relative proportions of fibers with dark and light color in ATPase-4.35 remained fairly constant during postnatal development (inset of Figs 4 and 5). This is confirmed with an analysis of variance. The area of the type II fibers (dark in ATPase-fix and light in ATPase-4.35) increased steadily up to

70 weeks of age followed by an accelerated increase between age 70 weeks and the adult stage. Type I fibers, which were light in ATPase-fix and dark in ATPase-4.35, increased linearly through the whole age span. Type IIC fibers, dark in both ATPase-fix and -4.35, were not identified and measured separately, since it was possible to trace fibers in both stains only in a few muscles of each age group. The general impression is that IIC fibers were smaller than types I and II fibers and similar in growth pattern to the type IIC fibers in the orbital layer. The proportion of the global type IIC fibers remained the same throughout development.

In the adult cat the type II fiber mean area was significantly larger than type I fiber area ( $P < 0.05$ ). At ages below 70 weeks there was no significant difference between the groups.

#### Orbital fiber fiber types and size changes

All fibers of the orbital layer stained darkly in ATPase-fix in muscles of all age (Fig. 6 inset).

Table 1 Suggested correlation between ultrastructural and histochemical types of muscle fibers in a cat extraocular muscles

Electron microscopical data from Alvarado & Horn (1975) and Alvarado et al (1978) and histochemical data from present study

EM type	1	2	3	4	5
Layer	Global	Global	Global + orbital	Global	Global + orbital
Mean diam ( $\mu\text{m}$ )	30	22	17	23	1
Membranous SR + TT	Much	Much	Moderate	Poor	Poor
Mitochondria	Few/small	Moderate	Many/large	Few	Many
Proposed innervation	Single	Single	Single	Multiple	Multiple
% of global fibers	38	70	76	15	
% of orbital fibers	-	-	60-70		30-40
Histochem type	Global II	Global II	Global + orbital II	Global I	Global + orbital IIC
ATPase-9.4	++	++	++	II	++
ATPase-fix	++	++	++	II	++
ATPase-4.35	0	II	0 +	++	++
SDH	0	+	++	0 +	++
% of global fibers	36	4	17	14	9
% of orbital fibers	-	-	54		46

Alvarado et al (1979) report occurrence of type 4 fibers in orbital layer and types 5 fibers in global layer but not relative proportions of each type

About 80% of the fibers reversed their staining properties and became pale in ATPase-4.35 being ordinary type II fibers. The rest of the fibers were dark also in ATPase-4.35 i.e. they were type IIC

The proportions types II and IIC fibers do not change significantly with age (Fig. 7 inset). The growth patterns were similar in both types of fiber and there were no size differences between types any age. They changed size postnatally in a manner similar to global type II fibers exhibiting the marked size increase between 70 weeks and adult age after a linear increase up to 70 weeks.

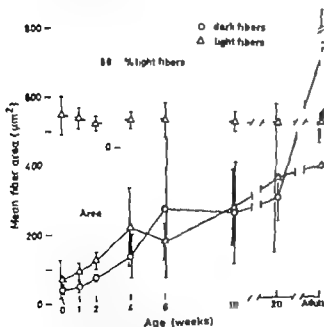


Fig. 4 Diagram showing the increase in mean area of global fibers staining darkly (type II and IIC) and lightly (type I) for ATPase-fix. The inset shows the proportion of light (type I) fibers at different ages. The bars indicate standard deviation in this and the following diagram.

## DISCUSSION

### Differentiation with age in fiber enzyme activity

This study has shown that cat eye muscle can differentiate into separate fiber types already at birth. The relative proportions of the various types did not seem to change with age although the growth patterns were different.

In the newborn kitten the fiber differences in staining properties with acid-stable and alkali-stable ATPase were as large as in the adult animal. Such differences in the activity of alkali-stable ATPase activity have previously been demonstrated in hindlimb muscle of newborn kitten (Karpati & Fiegel 1967; Nyström 1968) but not in rat and mouse muscles (Dubowitz 1963, 1965; Karpati & Fiegel 1967). Less distinct differences in alkali-stable

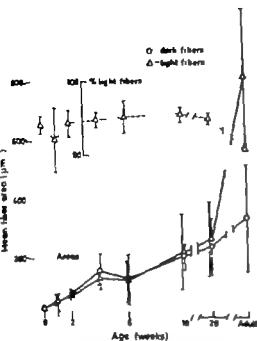


Fig. 5. Graphs to show increase of mean fiber area with age in the globe layer stained for ATPase-4.35. The dark fibers are type I and IC; the light fibers type II. The inset shows the percentage of light fibers at different ages.

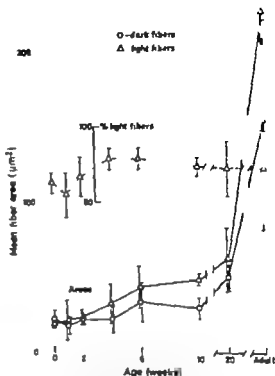


Fig. 7. Changes with age in orbicular fibers stained for ATPase-4.35. Dark fibers (type IC) and light fibers (type II) show the same growth patterns. In the inset the percentage of light fibers has been plotted.

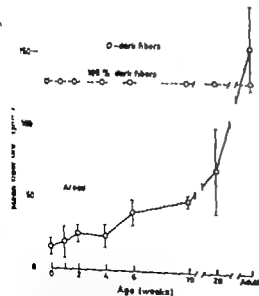


Fig. 6. Graphical presentation of fiber growth in the orbit layer. All fibers stained darkly for ATPase-6.6 (type II and IC fibers). Inset shows the percentage of dark fibers.

ATPase activity have been seen in new-born rabbits and hamsters (Dubowitz 1963, 1965). The length of the gestational period has been considered to be an important factor for the muscle fiber differentiation at birth (Dubowitz 1965). However, Ashmore & Doerr (1971) working with chicken demonstrated that the differentiation at hatching varied between muscles and was higher in leg muscles than in the pectoralis muscle.

Acid-stable ATPase activity of developing muscle has not been studied in leg or eye muscles. The present investigation showed that the differentiation was as high an acid-stable as in alkali-stable ATPase activity, which indicates that the development of these enzymes are governed by similar factors. We have also found differences between eye muscle fibers of the newborn kitten in SDH stains, although they were much smaller than in ATPase stains. There was a general increase in SDH-activity after two weeks of age and a further differentiation between fibers in SDH staining.



Tabl. 1 Suggested correlation between ultrastructural and histochemical types of muscle fibers in cat extraocular muscles

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ATPase-fix	++	++	++	0	++
ATPase-4.35	0	0	0	++	++
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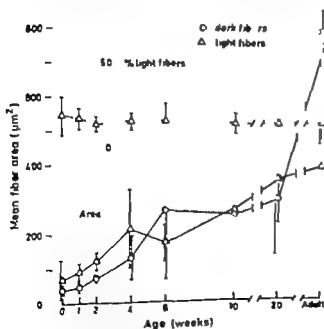


Fig. 4 Diagram showing the increase in mean area of global fibers staining darkly (type II and IIC) and lightly (type I) for ATPase-fix. The inset shows the proportion of light (type I) fibers at different ages. The bars indicate standard deviation in this and the following diagram.

fibers. The proportions types II and IIC fibers did not change significantly with age (Fig. 7 inset). The growth patterns were similar in both types of fiber and there were no size differences between types any age. They changed size postnatally in a manner similar to global type II fibers exhibiting the marked size increase between 70 weeks and adult age after a linear increase up to 70 weeks.

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ded that this staining pattern indicated multiple innervation: chicken anterior latissimus dorsi fibers with these histochemical properties have also been found in young rat skeletal muscle where they are thought to represent a transition form between types I and II fibers (Kugelberg 1973). In eye muscle this pattern was certainly not restricted to immature muscle fibers as suggested for skeletal muscle by Guth & Samaha (1972).

Slipply innervated fibers have been shown to have long lasting contractions following applications of succinylcholine (Sch) (Bach-y-Rita 1971). However the amount and also the total transverse section of type I and type IIC fibers was too small to allow us to account for the whole contraction after historesection, which usually reached 40–50% of the maximal tetanic tension (Lennernstrand & Hansson 1973). Thus, either some of the type II fibers are bimotone innervated or some slipply innervated fibers reacted with sustained contraction on application of Sch.

#### Correlation between histochemistry and electron microscopy

Electron microscopy has provided information on the correlation of eye muscle fiber structure and function (Lennernstrand & Nichols 1977; Bach-y-Rita et al. 1977) as well as the relationship of fiber structure to innervation (Alvarado et al. 1979). Histochemical studies, however, take less time and provide additional independent data on enzymes for assessment of fiber structure and function. Histochemistry could be especially useful for survey of fiber types throughout a single muscle. We have compared the cell types distinguished by histochemistry with those characterized by electron microscopy (Alvarado & van Horn 1975). The multiply innervated fiber types 4 and 5 have already been discussed. They are suggested to be equivalent to types I and IIC fibers, respectively. Type I fibers are found in the global layer and type IIC fibers in both layers.

Slipply innervated fibers of the electromicroscopical type 3 are small fibers found predominantly in the orbital layer. They are presumably equivalent to the orbital type II fibers in histochemistry. Type 3 fibers are rich in mitochondria (Alvarado & van Horn 1975) as are the type II fibers, staining darkly for the mitochondria-based enzyme SDH.

Slipply innervated types 1 and 2 fibers are found in the global layer together with type 3, 4 and 5 fibers

(Alvarado & van Horn 1975). Both type 1 and 2 are large fibers, type 1 being the largest but containing less mitochondria than type 2. Thus, type 1 fibers would seem to correspond best to the histochemical type II global fiber with very little SDH. The type 2 fiber would probably be the histochemical type II global fiber with higher SDH activity.

The information given on the correlation between electron microscopy and histochemistry of the different eye muscle types has been summarized in Table 1. Classification according to this scheme was difficult in younger cats because of less distinct layering and more random distribution of cell types, as well as smaller size differences between fibers. There were also cells in these preparations which could not be classified. It should be noted that young eye muscles have not yet been examined with the electron microscope.

#### Functional implications

Demands from the visual and oculomotor systems presumably have a strong influence on the development of cat eye muscles. During the sensitive period of visual development, lasting in the cat from three to about ten weeks of age (Hubel & Wiesel 1970; Blakemore & van Stryer 1974), motor activity for fixation and binocular fusion would seem of particular importance. These motor functions are probably best subserved by fatigue resistant fibers, for example of type I and IIC. When predatory skills and other complex motor patterns are exercised at a later age, increased demands for rapid refutations and saccadic eye movements can be expected. These might predominantly involve type II fiber activity. There would thus seem to exist functional justifications for developing slow fiber components earlier than the fast fibers as suggested from the physiological and histochemical results.

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properties took place. This agrees with previous findings in other kitten muscles (Nystrom 1968; Schwieler 1968; Hammarberg 1974).

#### *Correlation between physiology and histochemistry*

At all ages the eye muscles contracted much faster than other muscles (Lennerstrand & Hanson 1978a). This is consistent with the comparatively large amount of fast contracting type II fibers present in eye muscles already at birth. Type I fibers would constitute a slow contracting group of eye muscle fibers. The orbital type IIC fibers that stained equally dark for ATPase at pH 9.4 and 4.35 probably contract with intermediate velocity (cf. Kugelberg 1976). These findings are in agreement with the physiological differentiation of eye motor units by Lennerstrand (1974).

Experiments on twitch and tetanic tension development and on the effects of repetitive stimulation were performed on the same muscles that were examined histochemically (Lennerstrand & Hanson 1978a, b). Physiological results suggested that the slow fibers attained their adult properties already at age 6–10 weeks, while the fast fibers seem to reach their mature stage much later. Histological correlates to these differences in functional development might be found in the growth pattern of the 3 fiber types. Until the age of 10–20 weeks, fibers of all types were of almost equal size, but after this period growth was much faster in type II and IIC fibers than in type I fibers. A similar relationship has also been demonstrated in cat hindlimb muscles, where the muscle fiber area of the fast gastrocnemius muscle increased considerably more than the fiber area of the slow soleus muscle during the last part of the postnatal development (Eldred & Maier 1974). Since force of fiber contraction is directly related to fiber area, the large late increase in type II fiber size would explain the steady increase in speed of contraction seen in eye muscles up to adult age. The much smaller increase in size of the slow and presumably fatigue resistant type I fibers would be consistent with the finding that fatigue properties changes very little after 10 weeks of age.

The separate time courses for differentiation of ATPase and SDH activities of the muscle fibers indicate that the development of these enzymes are regulated separately. The ATPase is probably governed by neuronal factors other than impulse activity in the motor nerve, while the SDH level would

seem adjusted to the amount of work demanded by the muscle and regulated by the flow of impulse to the motoneurons (see for instance Riley & 1973). The opening of the eyelids at 2 weeks of age and the development of visual functions at 3 weeks would impose great demands for metabolic activity in some (not necessarily all) of the muscle fibers. A concomitant increase of the contraction velocity (Lennerstrand & Hanson 1978) probably also augmented the requirements for oxidative capacity in some of the eye muscle fibers (cf. Kugelberg 1973). The times at which the claims for fatigue resistance occurred correspond well with the observed increase in SDH activity from two weeks of age and onwards.

#### *Histochemical properties of multiply innervated fibers*

Cat eye muscles contain fibers with multiple innervation in addition to the singly innervated fibers commonly seen in skeletal muscle (Hess & P. 1963; Lennerstrand 1974; Alvarado & van Horn 1975; Alvarado et al. 1979). The multiply innervated fibers of eye muscles are known to be of two types. One of them is the fiber in the global layer with dense multiple innervation resembling multiply innervated slow fibers in amphibian muscle. They were called type 4 fibers in the classification of electronmicroscopy by Alvarado & van Horn (1973). It is suggested that the type 4 fibers correspond to the histochemical type I fibers found in the global layer in this study. Type I fibers contract slowly and are pale in SDH, which is also characteristic of amphibian slow multiply innervated fibers (Lännergren & Smith 1966; Smith & Lännergren 1968; Smith & Ovalle 1973).

The second type of multiple innervation is represented by the orbital fiber of type 5 in the classification of Alvarado & van Horn (1975). The endplates are more widely distributed than on type 4 fibers. Similar fibers have been found in avian muscles. It has been speculated that the fibers with both alkaline stable and acid stable ATPase, type IIC fibers, are multiply innervated fibers (Jellin 1969; Hanson & Lennerstrand 1977). The decision that the fibers that stained positively in both acid and alkaline pre-incubated ATPase and strongly in SDH are equivalent to the type 5 fibers in the classification of Alvarado & van Horn is further supported by findings of Amussen, Kieselring & Wohlrab (1971) and A. H. Moore & Doerr (1976), who have con-

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# Role of heating in non-invasive blood pressure measurements in rats

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BORG E. & VIBERG A. Role of heating in non-invasive blood pressure measurements in rats. *Acta Physiol Scand* 1980; 108: 73-75. Received 9 May 1979. ISSN 0001-6772. Department of Physiology II, Karolinska Institute, Stockholm, Sweden.

The 'tail-cuff' technique for indirect blood pressure measurements was standardized with respect to heating, in experiments on 47 adult Sprague-Dawley rats. The direct, intra-arterial and the indirect blood pressures were simultaneously obtained. Pulse volume was recorded from the tail by non-invasive technique, and was controlled by gradual application of heat. It was found that pulse volume and body temperature were poorly related. A stable relation existed, however, between the error in the indirect blood pressure recordings and the pulse volume. The error was minimized, provided that the heating was adjusted to induce pulse volume of at least 25% of the maximum obtainable value. It was suggested that the validity of the indirect blood pressure recording could be improved, if heat application was regulated on the basis of observations of pulse volume in the tail instead of ambient temperature or body temperature. Errors due to excessive vasoconstriction or discomfort due to overheating could thereby be minimized.

**Key words:** Blood pressure, rats, temperature regulation, tail.

Non-invasive measurements of blood pressure in animals are commonly made by utilization of the 'tail-cuff' technique. In principle, the technique is equivalent to the one used in humans. The bloodflow is occluded by compression of the tail and the systolic pressure is defined as the level at which out-flow is just detectable peripheral to the occluding cuff upon relieving the cuff pressure. The difficulty for detecting a minimal flow is therefore crucial to the precision of this technique.

One major technical difficulty and an important source of measurement error and of data misinterpretation is associated with the recording of out-flow in the tail. At room temperature the tail arteries are constricted, the flow is very low and the recording usually has a poor signal-to-noise ratio. Under such conditions flow will not be detected until the cuff pressure is significantly below the true systolic pressure. In addition, a pressure drop will occur in the tail vessel from its origin at the aortic bifurcation to the point of measurement, 2-4 cm to the periphery. In order to circumvent these difficulties, the 'tail-cuff' technique involves standardized heating. The blood-flow in the tail is thereby increased about hundredfold

(Johansen 1962). Heating can obviously only make an adequate blood-flow in the tail more or less probable, but it cannot ensure such conditions (Sobin 1946; Bunag et al 1971). Furthermore, the procedure might cause overheating, discomfort and possibly itself induce a rise in blood pressure (Proskauer et al 1945).

In order to avoid some of the difficulties associated with heating, it would be advantageous to be able to establish a set of criteria for adequate measuring conditions based on observations on blood-flow in the tail made simultaneously with the blood pressure recording.

The aims of the present study have been to:

- determine the relation between body temperature and pulse volume in the tail (an indirect measure of blood-flow; Hellström 1973)
- determine how the error in the indirect blood pressure reading varies as a function of pulse volume in the tail.

## METHODS

The total of 47 Sprague-Dawley albino rats, 150-620 g, were used in acute experiments relating blood pressure to



als tested underwent a period where body temperature exceeded  $38^{\circ}$  in spite of having a vasoconstriction with a pulse volume less than 20% of minimum. It is also to be noted that large amplitude waves were recorded at normal and sub-normal temperatures at the end of the experiment. A clear relation between body temperature and pulse volume was a typical finding.

**Validity of indirect blood pressure measurements.** The direct and indirect blood pressure were compared at various pulse volume amplitudes in under anaesthesia. Fig. 2 shows the relation  $IND \times 100\%$  between the direct systolic ( $D$ ) and indirect ( $I$ ) pressures as a function of relative pulse volume measured immediately before the inflation of the cuff. An exponential function was fitted to the experimental values by a non-linear regression technique. It is seen that below about 10% of maximum of pulse volume, the indirect values are variable and usually much below the direct pressures. Above approximately 25% the relation between indirect and direct measures was no further improved. Regression lines were calculated for all values above 20% for each tail. Mean slope of these lines was close to zero (0.0) S.D. = 0.078  $N=7$ ). In most cases a static or remained which amounted maximally to 20% of the intra-vital pressure values. As the same cuff was used on all animals irrespective of animal size, the error is to be expected (see e.g. Fregly 1963). The relation between this steady state error in pulse volume was large) and the body weight of the animal was determined for 36 animals. The large animals (males) had a higher blood pressure in the tail (indirect  $\bar{x}=5.3\%$  S.E.=2.2) than in femoral artery. Small animals (most of them males) had a 8.6% (S.E.=3.3) lower indirect than direct pressure.

## CONCLUSIONS

The present results show that:

- 1 The pulse volume (blood-flow) is not a simple function of body temperature.
- 2 The animal has to be heated to give a pulse volume in the tail of at least 70–85% of the maximum in order to allow adequate indirect blood pressure readings.
- 3 An error remained (with 20 mm cuff) in spite of heating. The indirect pressure in full-grown male rats was 5–10% higher than the direct pressures whereas it was 5–10% lower in females.
- 4 Pulse volume recorded from the surface of the tail is more reliable than body temperature or ambient temperature for standardization of measuring conditions when utilizing the "tail-cuff" technique.

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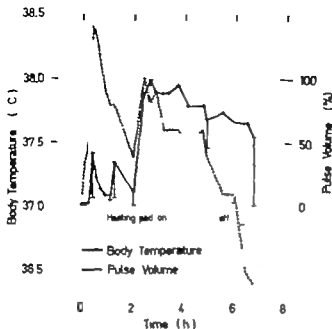


Fig. 1 Typical example of relative pulse volume in the tail and rectal temperature various times after initiation of heating and during subsequent cooling of a non-anesthetized rat. Vertical bars indicate range of pulse volume values during a 5-min observation period.

arterial pulse volume and pulse volume to body temperature.

**Recording of pulse volume and blood pressure.** The blood-flow in the tail was indirectly assessed by surface recorded arterial volume pulsations (Hellström 1973). A rubber balloon was taped to the ventral side of the tail and connected to an Elema 510C transducer whose signals were amplified and recorded on an Elema T 34 recorder. Blood-flow was occluded at the base of the tail by an inflatable cuff (12×70 mm lined with latex rubber). The pressure in the cuff was raised rapidly to suprasystolic levels and then gradually released. The cuff pressure measured by a modified commercially available system (TechLab) was monitored on the recorder and on a standard sphygmomanometer. The i.a. blood pressure was obtained by connecting the iliac artery to a Statham P23 DC transducer with a PE 50 or PE 60 tube.

The animals were heated either by a temperature-controlled heating-pad (Antemp Jacoby, Sweden) or by a conventional light bulb. Rectal temperature was continuously monitored on a galvanometric thermometer (Elektrilaboratoriet, Copenhagen).

**Procedure.** For comparison of direct and indirect blood pressures, the animals were kept in pentobarbital anaesthesia and heated to produce various amplitudes of pulse volume (blood-flow). Simultaneous readings of direct and indirect pressure were made at widely different values of pulse volume. The maximum pulse amplitude was obtained after considerable overheating of the animals. For comparison between body temperature and tail pulse volume, non-anesthetized animals were used. The rats were adapted to the measuring situation during a 3-day training period. During the experimental sessions, the rat was ini-

tially anesthetized with ether and placed on the rep. heating-pad in the wiremesh tube used for respiration. Rectal temperature and pulse volume were continuously recorded on the non-anesthetized animal during heating and subsequent cooling over a total period of 4–8 h.

## RESULTS

**Relations between body temperature and pulse volume in the tail.** In the measurements on anesthetized animals, the relation between body temperature and pulse volume was variable. Rectal warming was more efficient in increasing pulse volume (in spite of low body temperature) than the usage of the heating pad. These observations may however not be relevant for blood pressure measurements with the tail-cuff technique. Such measurements are regularly made on non-anesthetized animals.

In the experiments on non-anesthetized rats, body temperature and pulse volume could be more adequately compared. Fig. 1 shows a typical example of rectal temperature and relative pulse volume as a function of time after start of the experiment. It is seen that the body temperature was initially 37°C and that it could be raised to 38°C without being accompanied by any appreciable increase of pulse volume. When the vessel dilated, body temperature usually dropped almost to normal and then increased again. A long period of variable pulse volume (vertical bars) is observed in Fig. 1 before steady maximum dilatation occurs. Seven of the

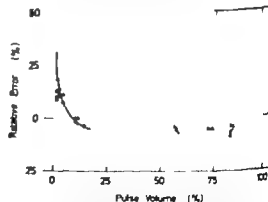


Fig. 2 Relative difference between intra-arterial and cuff pressure in an anesthetized rat as a function of degree of pulse volume (blood-flow) in the tail. Ordinate:  $(\text{Direct} - \text{Indirect}) / \text{Direct} \times 100\%$ . Abscissa: Pulse volume amplitude in percent of maximum obtainable value.  $\square$  indicate observed values. Adapted exponential function (continuous line):  $y = a + be^{-x}$ .  $\sigma$  indicates steady state error (6%) and  $c = 0.19$ .

# ***β-adrenergic dilator interaction with the constrictor response in resistance vessels of skeletal muscle during hemorrhage***

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HILLMAN J & LUNDAVALL J. *β*-Adrenergic dilator interaction with the constrictor response in resistance vessels of skeletal muscle during hemorrhage. *Acta Physiol Scand* 1980, 108: 77-83. Received 14 May 1979. ISSN 0001-6772. Department of Physiology and Biophysics, University of Lund, Sweden.

A marked *β*-adrenergic dilator interaction with the vasoconstrictor response in skeletal muscle during hemorrhage is described. The dilator influence corresponded to some 40% of the constrictor response both at mild and moderate as well as at large bleeding. In absolute resistance units the *β*-adrenergic dilator influence averaged no less than 14 mmHg/dm<sup>2</sup> (ca. 100 g) at large bleeding (hemorrhagic hypotension of 50 mmHg). Comparison of the hemorrhage reduced resistance effects in the autoperfused denervated muscle, in the autoperfused denervated muscle and in the innervated muscle cross-circulated from donor animal showed that the *β*-adrenergic dilator influence more or less completely was caused by blood-borne catecholamines, in all likelihood by adrenaline which is known to be secreted in large amounts during hemorrhage. The described *β*-adrenergic dilator mechanism may serve to maintain intratissue blood flow by counteracting the constrictor response. It deserves consideration also from the point of view that it obviously has to be taken into account for proper evaluation of other vascular control mechanisms brought into action in hemorrhage.

Great effort has been devoted to the problem of vasomotion during hemorrhage of peripheral vascular resistance in hemodynamically important vascular areas. Skeletal muscle, with its large tissue mass, is one of these main targets for vasomotor responses in the body: reflex adjustment of blood flow during bleeding (see e.g. Chen 1967). The reason of muscle resistance during hemorrhage has been attributed to active vasoconstriction induced by the sympathetic vasomotor fibres and vasoactive hormones, e.g. the catecholamines, norepinephrine and vasopressin, and further to passive factors such as elastic recoil of the vascular wall, exposure to the decreased transmural pressure and rheological phenomena.

The vasoconstrictor response in muscle during hemorrhage has been reported to be modified by chemical dilator factors accumulating at

result of the reduced blood flow, especially during prolonged periods of more severe bleeding (e.g. Mellander & Lewis 1963). Apart from this little attention has been paid to the possibility that antagonistic dilator interaction with the vasoconstrictor influence may be of importance in determining the net resistance effect despite the fact that such possible interaction might have important functional implications, primarily with regard to tissue nutrition. A previous study performed on cat skeletal muscle (Lundvall & Hillman 1978) showed, however, that hemorrhage may be associated with quite a pronounced *β*-adrenergic inhibitory influence on vascular tone in the resistance vessels. In the present investigation this inhibitory resistance response was studied in more detail in order to evaluate its quantitative importance during graded hemorrhage and to reveal the mechanisms responsible for the reaction.



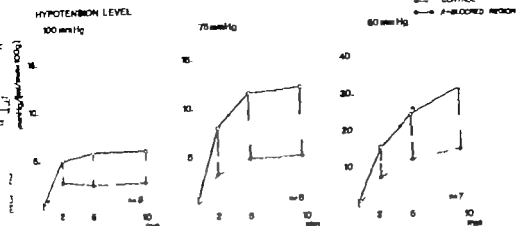


Fig. 1 Increase of muscle vascular resistance during graded hemorrhage in the autoperfused innervated regions with (○) and with blocked  $\beta$ -adrenoceptors (●). Statistical difference at  $P < 0.05$  and  $^{**} = P < 0.01$ .

in values  $\pm$  SE) refer to the autoperfused innervated, autoperfused denervated and cross-innervated innervated muscle regions with intact  $\beta$ -receptors ("intact" region) and with blocked  $\beta$ -receptors ( $\beta$ -blocked region) respectively as can be seen that resting vascular resistance averaged 16–17 mmHg/(ml  $\times$  min  $\times$  100 g) in the innervated intact region and 13 mmHg/(ml  $\times$  min  $\times$  100 g) in the denervated intact region. These values are normal for cat skeletal muscle (Landvall 1977). The values for vascular resistance in the  $\beta$ -blocked muscle region did not differ significantly from those in the intact region.

Fig. 1 summarizes the observed resistance changes in the autoperfused, innervated muscle in terms of the increase of regional resistance (mmHg/min  $\times$  100 g) above control level after 5–10 min at each hypotension level (mean values  $\pm$  SE, note the different scales along the ordinate). In the muscle region with intact  $\beta$ -adrenoceptors (open line) as well as in the  $\beta$ -blocked muscle region (solid line) the resistance response was related in relation to the degree of hypotension, but each hypotension level the increase of resistance or  $\beta$ -blockade showed a clear tendency to be more pronounced than in the muscle region with intact  $\beta$ -adrenoceptors (cf. significance values in the diagrams). This discrepancy implies that hemorrhage was associated with clear-cut adrenergic dilator influence in the resistance level of the autoperfused, normally innervated skeletal muscle vascular bed (the diagrams of

Fig. 2 this  $\beta$ -dilator effect is expressed as the difference between the resistance increase in the  $\beta$ -blocked and in the intact muscle region. It can be seen that the dilator influence was graded in relation to the degree of hemorrhage and after 10 min the  $\beta$ -dilator influence averaged 3.4, 6.9 and 13.9 mmHg/(ml  $\times$  min  $\times$  100 g) at the hypotension levels 100, 75 and 50 mmHg. During large bleeding (50 mmHg) the dilator effect thus indeed was pronounced. When expressed in relative terms however the  $\beta$ -adrenergic dilator influence was quite significant also at mild (100 mmHg) and moderate (75 mmHg) hemorrhage as indicated by the

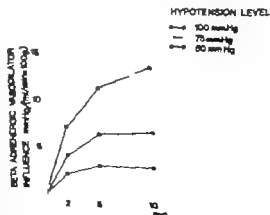


Fig. 2 Calculated  $\beta$ -adrenergic vasodilator influence in the autoperfused innervated muscle during graded hemorrhage.

Table 1 Mean values  $\pm$  S.E. in the control state before hemorrhage for arterial blood pressure,  $\pi$  blood flow and regional vascular resistance in the innervated autoperfused lower leg muscle region in the denervated autoperfused muscle ( $n=5$ ) and in the cross-circulated muscle region ( $n=6$ )

Paired and concomitant observations were obtained on the left lower leg muscles with intact and on the right leg muscles with blocked  $\beta$  adrenoceptors

	Innervated control region ( $n=8$ )	Denervated region ( $n=5$ )	Cross-circulated region	
			Recipient	Donor
<b>Intact region</b>				
Arterial pressure mmHg	133 $\pm$ 3	144 $\pm$ 3	141 $\pm$ 6	141 $\pm$ 1
Regional blood flow ml/min $\times$ 100 g	8.5 $\pm$ 0.4	9.4 $\pm$ 0.4	7.9 $\pm$ 0.7	
Regional resistance mmHg/(ml/min $\times$ 100 g)	16.9 $\pm$ 1.1	12.8 $\pm$ 0.4	16.9 $\pm$ 1.8	
<b><math>\beta</math>-Blocked region</b>				
Arterial pressure mmHg	136 $\pm$ 3	125 $\pm$ 3	141 $\pm$ 6	132 $\pm$ 1
Regional blood flow ml/min $\times$ 100 g	8.0 $\pm$ 0.5	8.9 $\pm$ 0.5	7.2 $\pm$ 0.5	
Regional resistance mmHg/(ml/min $\times$ 100 g)	17.4 $\pm$ 1.6	13.5 $\pm$ 0.4	17.1 $\pm$ 1.2	

## METHODS

The study was performed on 19 cats (7.6–3.8 kg) anaesthetized i.v. with chloralose (30 mg/kg b.wt.) and urethane (100 mg/kg b.wt.) after induction with ether.

Observations were made on two identical muscle regions viz. the right and left lower leg muscles which were isolated so that on each side the popliteal artery and vein formed the sole vascular connections with the main part of the body. The muscles were wrapped in gauze soaked in saline and covered by a plastic sheet to preserve a moist environment. Muscle temperature was maintained normal using a heating-lamp. After heparinization (750 IE/kg b.wt.) venous outflow from each muscle region was diverted from the popliteal vein to the right external jugular vein. An optical drop-recorder was inserted bilaterally in the venous shunt for continuous recording of the blood flow. A short shunt circuit connected the femoral and popliteal artery on each side and was used for regional administration of propranolol in order to block the  $\beta$ -adrenoceptors. The right brachial artery was cannulated and connected to a siliconized graduated pressure bottle the pressure of which could be adjusted to desired levels. Arterial blood pressure was monitored from a T-tube in the cannula in the brachial artery. Venous outflow pressure determined by the height of the orifice of the venous outflow tubing was maintained constant at 5 mmHg on both sides.

Changes of vascular resistance in response to hemorrhage were studied on the autoperfused innervated muscles, on the autoperfused denervated muscles and on the cross-circulated innervated muscle region. The sciatic nerve which contains virtually all sympathetic fibres to the lower leg muscles was left intact on both sides in 8 animals and severed bilaterally in 5 animals; both groups of animals being autoperfused. In 6 animals with intact sciatic nerves the lower leg muscles were supplied with blood from a donor cat by connecting with siliconized polyethylene tubings the femoral arteries of the donor to the popliteal arteries of the right and left lower leg muscle

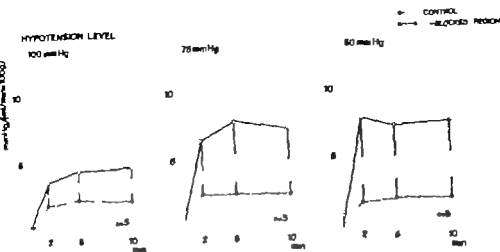
preparations of the recipient. The outflow of blood from the cross-circulated muscle region was passed by the optical drop-recorders and returned to the donor cat via the right external jugular vein.

Hemorrhagic hypotension was produced by rapidly bleeding into the pressure bottle. Stepwise graded bleedings to the three hypotension levels of 100 mmHg, 75 mmHg and 50 mmHg was performed. At each hypotension level arterial pressure was maintained constant for 10 min so that the total period of hemorrhage amounted to 30 min. After reinfusion of the shed blood the animals rest for another 30 min and were then subjected to a second identical period of stepwise bleeding. The doses of blood that had to be withdrawn to achieve the arterial blood pressure levels of 100, 75 and 50 mmHg was quite similar in the autoperfusion and cross-circulation experiments, on average it amounted to 7.15 and 6.1 ml/kg b.wt. During sequences of stepwise bleeding, the right lower leg muscle preparation was subjected to regional blockade of  $\beta$ -adrenoceptors by propranolol whereas the  $\beta$ -adrenoceptors of the muscle preparation on the left leg was intact. The  $\beta$ -blocking agent was administered distally into the arterial shunt circuit in a dose of 100  $\mu$ g. Concomitantly venous outflow of blood was sampled and discarded to avoid systemic effects of the blocking agent. The discarded blood was substituted by administration of the same volume of dextran.

In Results section each prevented observation reached by calculating the mean of the two responses obtained in the separate periods of bleedings from the same animal. Spread of data is expressed as S.E. significance test were performed according to Student's  $t$  test.

## RESULTS

Table 1 gives data on arterial blood pressure, regional blood flow and regional vascular resistance in the control state before bleeding. The



1 Increase of regional vascular resistance during graded hemorrhage in the autoperfused denervated muscle with and without blocked  $\beta$ -adrenoceptors. — statistical difference at  $P < 0.05$  and — at  $P < 0.01$ .

that bleeding is associated with a marked adrenergic dilator influence on the resistance of skeletal muscle. In absolute resistance the dilator influence was found to be related to the degree of hemorrhage and at large bleeding effect reached no less than 14 mmHg/(ml  $\times$  100 g). When calculated in relative terms dilator influence was marked at all levels of drug. Thus it was deduced to correspond to c. 40% of the constrictor response both at light moderate as well as at large bleeding (Fig. 3). Functional significance of such an inhibitory effect on vascular tone remains to be clarified. Perhaps primarily serve as mechanism for resistance of outflow blood flow during the hemorrhage induced vasoconstriction. However described dilator mechanism deserves consideration also from the point of view that it has to be taken into account for proper evaluation of other vascular control mechanisms brought into action in hemorrhage.

In three different series of experiments performed on the autoperfused innervated muscle on autoperfused denervated muscle and on the unperfused innervated muscle respectively it is clear that the observed  $\beta$ -adrenergic dilator influence more or less completely was caused by blood-borne catecholamines, in all likelihood by adrenaline which is known to be released in large amounts during hemorrhage (e.g. Watt, Hall & Hodge 1971; Jakobschik et al. 1974). Analogous adrenaline effect on muscle resistance

was reported by Chalmers et al. (1966) in rabbit subjected to severe hypoxia. The fact that the  $\beta$ -adrenergic dilator response was significantly smaller in the denervated than in the innervated autoperfused muscle at marked hypotension (50 mmHg) may not necessarily contend with this conclusion. It is known for example that the magnitude of a vascular effect often depends on the prevailing level of vascular tone (e.g. Myers & Honig 1969; Lundvall 1977), and this level differed markedly in the mentioned two preparations. The lack of any significant direct, nerve mediated  $\beta$ -adrenergic decrease of muscle vascular resistance during the hemorrhage induced reflex increase of sympathetic nerve discharge as demonstrated by the cross-circulation experiments is in accordance with a previous study using artificial electrical activation of the sympathetic nerves to skeletal muscle (Lundvall & Järhult 1976). These stimulation experiments failed to reveal any neurogenic  $\beta$ -adrenergic dilator effect on total muscle vascular resistance although there were a quite marked microvascular dilator influence. The blood-borne catecholamine induced  $\beta$ -adrenergic inhibition of vascular tone during bleeding which entirely must represent a regional effect in the muscle vascular bed, probably reflect direct interaction with specific receptors of the vascular smooth muscle cells. Indirect effects secondary to adrenergic alterations of the skeletal muscle metabolism can not be excluded however. Possible interaction with presynaptic  $\beta$ -adrenoceptors facilitating transmitter

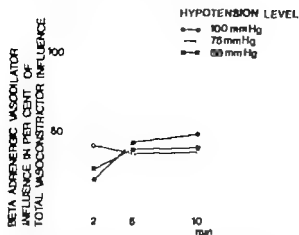


Fig. 3  $\beta$ -Adrenergic vasodilator influence in the autoperfused innervated muscle during graded hemorrhage in relation to the concomitant vasoconstrictor influence

data in Fig. 3. The extent to which the  $\beta$ -adrenergic dilator influence counteracted the simultaneous constrictor influence has here been deduced in rough terms by dividing the  $\beta$ -dilator effect (Fig. 2) with the total resistance increase observed in the  $\beta$ -blocked muscle region (Fig. 1). The data suggest that the constrictor response was reduced by some 40% as a result of the  $\beta$  adrenergic dilatation at all three levels of bleeding.

It would seem likely that the observed  $\beta$ -adrenergic dilator influence in the autoperfused innervated muscle during bleeding was exerted by blood-borne catecholamines released from the adrenal medulla but it may also be related to a  $\beta$  adrenergic dilator component of the vascular response evoked by the adrenergic vasomotor fibres (cf. Discussion). The latter possibility however seems refuted by the results obtained in experiments in which the innervated lower legs were cross-circulated from a donor animal (see Methods). In these experiments the concomitant resistance responses during bleeding of the recipient to 75 mmHg were followed in the left limb with intact  $\beta$  adrenoceptors and in the  $\beta$ -blocked right limb. The results are presented in Fig. 4 which shows that the evoked increase of resistance was very similar in the intact and  $\beta$  blocked muscle region.

The  $\beta$ -adrenergic dilator influence observed in the autoperfused innervated muscle (Figs. 1 and 2) thus seems attributed to the action of blood borne catecholamines. In an attempt to further elucidate such a humoral dilator effect the resistance function was studied in the autoperfused denervated muscle region during graded bleeding to the stand-

ardized hypotension levels of 100, 75 and 50 mmHg. The collected results from these experiments shown in Fig. 5 (mean values  $\pm$  S.E.). In the muscle region hemorrhage evoked an increase in resistance which was fairly small and of a magnitude at the different hypotension levels 3-8 mmHg/(ml  $\times$  min  $\times$  100 g $^{-1}$ ). Also in the  $\beta$ -blocked muscle region the response was roughly similar to the three levels of hypotension but resistance clearly more raised than in the intact muscle, 5-8 mmHg/(ml  $\times$  min  $\times$  100 g $^{-1}$ ). These experiments thus directly reveal quite a marked  $\beta$ -adrenergic dilator influence during hemorrhage. The magnitude of this dilator effect in the denervated muscle as reached by subtracting the resistance response in the intact region from that obtained in the  $\beta$ -blocked region amounted to some 3 mmHg/(ml  $\times$  min  $\times$  100 g $^{-1}$ ) at mild hemorrhage or about 5 mmHg/(ml  $\times$  min  $\times$  100 g $^{-1}$ ) at mild and large hemorrhage. The dilator effects at small and moderate bleeding are of similar magnitude to those presented in Fig. 2 for the experiments in the autoperfused innervated muscle where the  $\beta$ -adrenergic dilator effect at large hemorrhage (75 mmHg) was much more pronounced in the innervated than in the denervated muscle (see Discussion).

## DISCUSSION

The present study which confirms and extends previous observations (Lundvall & Hillman 1978)

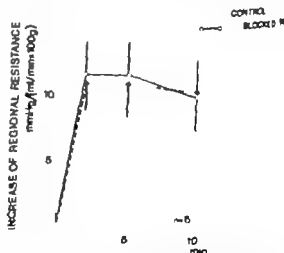


Fig. 4 Increase of muscle vascular resistance during hemorrhagic hypotension (75 mmHg) in the cross-circulated lower leg muscle with intact and with blocked adrenoceptors.

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release from the adrenergic nerve endings during nerve discharge (cf Langer 1977) also deserves consideration especially since this mechanism may be activated by blood-borne catecholamines (e.g. Stjärne & Brundin 1976). It can be deduced that blockade of any such facilitation in fact would lead to an underestimation of the magnitude of the post synaptic effector cell located  $\beta$  adrenergic inhibitory effect on vascular tone.

As mentioned previously, skeletal muscle is one of the tissues that contribute in an important way to the regulation of overall peripheral resistance during hypovolaemia (for ref. see Chien 1967). Many studies indicate that the evoked vasoconstriction mainly is due to increased discharge in the sympathetic vasoconstrictor fibres (e.g. Öberg 1964, Lundgren, Lundvall & Mellander 1964, Haddy, Scott & Molnar 1965, Bond, Manley & Green 1967). The blood-borne catecholamines have been proposed to contribute more or less markedly to the constrictor response (Bond, Manley & Green 1967, Hall, Schwinghamer & Lalone 1976) and vasoactive amounts of vasopressin (e.g. Rocha E. Silva & Rosenberg 1969, Cousmenu, Gagnon & Sirois 1973) and angiotension (cf Gutmann et al. 1973) may be released as well. Experimental evidence clearly indicate that species differences can be quite marked and further that the relative role of different vasoconstrictor stimuli varies with the type of bleeding performed (e.g. rapid or slow exsanguination) and with other methodological factors such as whether pressure bottle was used or not. The importance of the last mentioned factor for example may be demonstrated by the findings that the  $\alpha$ -adrenergic constriction as well as the  $\beta$  adrenergic dilatation increased significantly with time during large bleeding (Figs. 1 and 2) whereas both these responses soon reached a steady state in similar experiments without pressure bottle (unpublished data). This difference may be related to the greater stress the animal encounters in the pressure bottle experiments insofar that they can not benefit to full extent from the cardiovascular compensatory mechanism brought into play. As stated above, proper evaluation of the nature and magnitude of vasoconstrictor mechanisms also requires that possible and quantitatively important antagonistic dilator actions can be taken into account. The marked  $\beta$  adrenergic dilator influence revealed in this investigation is obviously one such factor and the necessity to account for it may be

demonstrated by the results obtained in the evoked autoperfused muscle preparation (Fig. 3). The resistance effects observed in the  $\beta$  preparation with intact  $\beta$ -adrenoceptors give the impression of a weak humoral vasoconstrictor influence. Yet the observations in the  $\beta$ -adrenoceptor blocked preparation reveal the presence of a quite pronounced hormonal vasoconstriction dominating. This vasoconstrictor influence seems caused by activation of  $\alpha$ -adrenoceptors and can be abolished after administration of  $\alpha$ -blockers (unpublished observations).

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# The course of adaptation to low intensity training in sedentary men: dissociation of central and local effects

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ÖRLANDER J, KIESSLING K H & EKBLOM B. Time course of adaptation to low intensity training in sedentary men: dissociation of central and local effects. *Acta Physiol Scand* 1980; 108: 85-90. Received 22 May 1979. ISSN 0001-6772. Institute of Zoophysiology, University of Uppsala, Department of Animal Nutrition, Swedish University of Agricultural Sciences, Uppsala, and Department of Physiology III, Karolinska Institute at Gymnastik och idrottshögskolan, Stockholm, Sweden.

The oxygen transporting capacity and the metabolic capacity of the vastus lateralis muscle were followed in parallel in 9 sedentary, overweighted men during a low intensity training program. Measurements were made at 0, 3, 6, 9, 1 and 30 weeks. Maximal oxygen uptake increased in an approximately linear fashion during the first 1 week (11%), but decreased little (3%) during the following 18 weeks. Mean body weight decreased 8% (7.4 kg) during the training. The distribution of muscle fibre types, including the subgroups of type II fibres, did not change. Muscle enzyme activities remained essentially unchanged during the training. It was concluded that 'central' and 'local' adaptation need not occur in parallel, and that the leg oxygen utilization capacity probably does not limit the whole body maximal oxygen uptake.

**Key words:** Low intensity training, muscle metabolism, oxygen uptake, time course.

Intensified training of a kind that can be carried out unsupervised by previously sedentary people has been shown to elicit physiological adaptation at the systemic as well as at the muscle level (e.g. Rodhe 1971; Pollock 1973; Örlander et al. 1977). In previous investigation (Örlander et al. 1977) we found that the increases in maximal oxygen uptake ( $\dot{V}_{O_2}$  max) and muscle metabolic potential were asynchronous and probably not directly related. Furthermore it appeared that the time course of the training response might be different for different metabolic pathways in muscle. The present study was undertaken as an attempt to clarify these points by following the time course changes in  $\dot{V}_{O_2}$  max and skeletal muscle enzymes in sedentary, overweighted men during a low intensity training program.

## SUBJECTS AND METHODS

**Subjects:** 9 clinically healthy, sedentary and overweighted men volunteered to participate in this study. An oral consent was obtained from each subject after he had been informed of the procedure and possible risks of the experiment. They were all unmarried, had white-collar jobs and had never been engaged in any regular physical training. Pertinent anthropometrical data (mean, S.D. range) for the whole group were: age 33±4 (22-35) years, height 183.7 (174-193) cm and weight 97.6±9.4 (84.7-114.0) kg. Five were smokers and 4 nonsmokers, but 3 of the smokers stopped smoking during the training period.

**Training regimen:** The first part of the training period lasted 12 weeks. During this period of time the subjects trained on their own about 4 km 5 times/week and had

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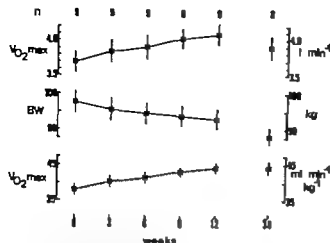


Fig. 1 Maximal oxygen uptake ( $\dot{V}O_2 \text{ max}$ ) and body weight (BW) at various times during the training (means  $\pm$  S.E.). The number of subjects at each test occasion is given at the top. All means at weeks 3–30 are significantly different from the 0 week means ( $P < 0.01$ – $0.001$ ).

weekly contact with the investigators. Training intensity was decided from the pre-training tests (see below) and aiming at an average oxygen uptake ( $\dot{V}O_2$ ) during the training workouts of 60–80 per cent of maximum. During the first 6 weeks the training consisted of "fast walking". During the following 6 weeks 1 or 2 of the walking workouts included some slow jogging (for definition of different exercise speeds and corresponding average load on oxygen transport see Ekblom et al. (1979)). The subjective rate of perceived exertion (RPE) corresponding to 80 per cent of  $\dot{V}O_2 \text{ max}$  was determined according to the Borg scale (1962) during the exercise tests. The subjects were asked not to exceed this RPE during training.

From 12 weeks on the subjects still trained on their own but had no contact with the investigators until the final test after 30 weeks. In this training period of 18 weeks the subjects were asked to continue with the fast walking-slow jogging program. The average attendance of training during this period was 3 times a week. One subject could not participate in the 30-week follow-up since he had moved abroad.

**Exercise tests.** Preliminary tests were performed to familiarize the subjects to the test methods used and to obtain preliminary data on  $\dot{V}O_2 \text{ max}$  and maximal work loads. Before the training started at each 3rd week up to the 17th week and after 30 weeks the same test procedure was applied. Each subject was weighed early in the morning after a light meal. With ECG electrodes attached he performed 1 or 2 submaximal 6 min work periods on a mechanically braked Monark bicycle ergometer and after a short warming-up period an exhaust run (work time 4–6 min) on a motor-driven treadmill. On another test day before the training started and at the 6th and 17th weeks test the subjects performed 6 min exercise period at 4 different speeds which were subjectively determined namely: normal walk, fast walk, slow jog and normal jog (for details see Ekblom et al. 1979). The latter tests were used for the prescription of the training speed. Since this paper deals with the "central" and "local" adap-

tation to a low intensity training program, the data from this part of the study will be published elsewhere.

On all work loads  $\dot{V}O_2$  was determined between 5 and 6th min of the submaximal work and at least 3 measurements were made towards the end of the test using the Douglas bag-Haldane technique. Heart rate (HR) was calculated from the ECG recorded continuously during submaximal and continuously at the end of maximal exercises.

The subjects were asked not to change the daily habits except excluding alcohol and between-meal snacks. However it cannot be excluded that some of them stopped smoking; some of the subjects changed the quality and quantity of the caloric intake.

**Muscle analyses.** Biopsies were taken from the vastus lateralis muscle with a needle technique (Bergström) alternating between the right and the left leg. The specimens were used for enzyme assays and for histology.

For enzyme activity determination homogenates were prepared and assays performed as described previously (Örlander et al. 1977) except that assays were run at 37°C. The investigated enzymes chosen to represent the pathways in energy metabolism were phosphofructokinase (PFK, E.C. 2.7.1.11), lactate dehydrogenase (LDH, E.C. 1.1.1.27), 1-hydroxyacyl-CoA dehydrogenase (HAD, E.C. 1.1.1.35), citrate synthase (CS, E.C. 4.1.3.1) and cytochrome oxidase (cyto c, E.C. 1.9.3.1). PFK activity was estimated according to Shonk & Boyer (1967), LDH and HAD by the method of Bassett et al. (1966), CS described by Srere (1969) and cyto c according to Wikström et al. (1969). Muscle protein was estimated by the method of Lowry et al. (1951).

For histochemistry a muscle specimen was uniaxially oriented, mounted in Cryofilm™, frozen in nitrogen, cooled by liquid nitrogen and stored at -80°C. Transverse sections (10 µm) were cut on a cryostat and stained for myofibrillar ATPase after alkaline (pH 10.3) or acid (pH 4.3–4.6) pretreatment (Padykula & Herman 1953; Guth & Samaha 1964; Bassett & Kahner 1970). Fibres were classified as type I (fast twitch) or type II (fast twitch) (Engel 1962). In biopsies type II fibres were subdivided into IIA and IIC (Brooke & Kaiser 1970; Dubowitz & Brooks 1973). An average of 154 fibres were counted per fibre. The variability in percent type I fibres between two specimens taken from the same subject was 8% (mean  $\pm$  S.D. calculated from 86 pairs).

Statistical evaluation of changes and inter-group differences was made with the paired and independent *t*-tests respectively. The level of significance was selected  $P < 0.05$ .

## RESULTS

$\dot{V}O_2 \text{ max}$  increased in an approximately linear fashion during the first 17 weeks of training reaching a value of 10.8 l min<sup>-1</sup> above the pre-training level ( $P < 0.001$ ) (Fig. 1). A significant (4.1 l min<sup>-1</sup>,  $P < 0.01$ ) increase was observed already in 3 weeks. Because the mean body weight decreased, there was a pro-

1. Distribution of muscle fibre types in vastus lateralis at various times during the training are shown percentages  $\pm$  S.E. The number of subjects is given within brackets. *P* values refer to the difference compared with the 0 week value.

Week of training	0	3	6	9	12	30
3	1.6 $\pm$ 0.9 (9)	29.5 $\pm$ 4.3 (9)	3.1 $\pm$ 6.6 (7)	32.3 $\pm$ 7.0 (5)	27.6 $\pm$ 4.0 (7)	26.9 $\pm$ 2.3 (6)
		>0.40	>0.70	>0.50	>0.30	>0.90
33.7	7.3 (7)				30.4 $\pm$ 4.9 (5)	30.8 $\pm$ 2.6 (5)
					>0.30	>0.40
44.3	6.1 (7)				4.4 $\pm$ 6.0 (5)	41.7 $\pm$ 4.2 (5)
					>0.60	>0.30
21.7	7.8 (7)				26.9 $\pm$ 4.7 (5)	27.2 $\pm$ 1.8 (5)
					>0.60	>0.70
0.3	0 (7)				0.4 $\pm$ 0.2 (5)	0.3 $\pm$ 0.3 (5)
					>0.90	>0.90

relative improvement in  $\dot{V}_{O_2}$  max per kg body weight. At 1 week a 16.6% increase ( $P < 0.001$ ) was seen (Fig. 1). In the 30 week test  $\dot{V}_{O_2}$  max in remaining 8 subjects had decreased 3.0% ( $P < 0.01$ ) from their 1 week mean value but was significantly higher than before training (8.0% ( $P < 0.01$ )). Because body weight continued to decrease  $\dot{V}_{O_2}$  max per kg body weight remained unchanged (Fig. 1). Those subjects with the lowest  $\dot{V}_{O_2}$  max tended to show the greatest absolute increases at 1 week. There was however no significant correlation ( $r = -0.47$ ,  $P > 0.20$ ).

The mean percentage of type I fibres in the vastus lateralis was 32.1% before training, and no significant change was seen during the training. Neither was there any change in the distribution of type II groups (Table 1). For technical reasons, biopsies could not be taken in some instances, and some analyses could not be analyzed. However virtually all results were obtained when only subjects who could be analyzed at all time points were considered. Furthermore statistical evaluation was based on individual comparisons only. The same reasoning applies to the enzyme activity data.

As shown in Fig. 2, muscle enzyme activities were essentially unchanged during the course of the study. At 6 weeks, LDH was lowered ( $P < 0.05$ ), and the same was true for CS at 12 weeks ( $P < 0.01$ ). A tendency ( $P < 0.10$ ) towards increased cytochrome *c* was evident at 12 weeks. No significant changes in muscle protein concentration were observed.

Correlations between the studied variables were

computed, and the following significant relationships were obtained: % type I vs.  $\dot{V}_{O_2}$  max at 6 weeks ( $r = 0.76$ ); % type I vs. cytochrome *c* at 3 weeks ( $r = 0.68$ ); % type I vs. PFK at 12 weeks ( $r = 0.84$ ); CS vs. HAD at 3, 9, 12 and 30 weeks ( $r = 0.82-0.98$ ). No significant correlation between % type I fibres and the increase in  $\dot{V}_{O_2}$  max was observed.

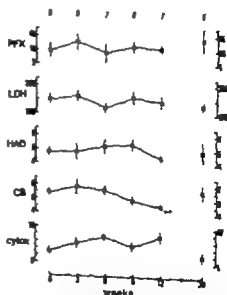


Fig. 2. Enzyme activities in vastus lateralis at various times during the training (means  $\pm$  S.E.). Numbers of subjects are indicated at the top. Activities are given as  $\mu$ mol/min (g wet wt<sup>-1</sup>) for phosphofructokinase (PFK), lactate dehydrogenase (LDH), 3-hydroxyacyl-CoA dehydrogenase (HAD) and citrate synthase (CS), and as  $\mu$ mol O<sub>2</sub>/min (g wet wt<sup>-1</sup>) for cytochrome oxidase (cytochrome *c*). \* and \*\* denote  $P < 0.05$  and  $P < 0.01$  respectively when compared with the 0 week value.

Pre training data for smokers ( $n=5$ ) and nonsmokers ( $n=4$ ) were not significantly different except for a higher ( $P<0.01$ ) percentage of type II A fibres in the smokers. Non-significantly lower mean values for  $\dot{V}_{O_2}$  max and % type I fibres were seen in the smokers ( $3.51$  vs  $3.91$  l·min<sup>-1</sup> and  $27.1$  vs  $38.4$ % respectively). Since 3 of the smokers quit smoking during the study no meaningful comparison of the training effects in smokers and nonsmokers was possible.

## DISCUSSION

In the present study the capacity of the oxygen transport system and the metabolic capacity of the vastus lateralis muscle were followed in parallel during a low intensity training program. The maximal oxygen uptake increased considerably while the skeletal muscle characteristics remained essentially unchanged (Fig. 1 and 2, Table 1). This indicates that the type, intensity and duration of training is of great importance for whether "central" and local adaptation will occur in parallel or not.

The increase in maximal aerobic power in the present study was similar to that in other low intensity training studies (Kilbom 1971, Örlander et al 1977). An initial increase rate of about one percent per week appears to be typical for this kind of training. It should be noted that due to the simultaneous weight reduction there was in the present subjects a substantial increase in  $\dot{V}_{O_2}$  max per kg body weight.

Evidently the increased metabolic demands due to the employed training program were within the capacity of the vastus lateralis muscle (Fig. 2). The decreased LDH activity at 11 weeks may be a chance finding. Such a decrease is however regularly observed during extensive endurance training (Holloszy & Booth 1976, Sjödin 1976). CS showed a decreasing trend from week 3 and was significantly lowered at 17 weeks. The reason for this is not clear. One could speculate that other factors than the training such as the changed dietary habits might play a role. It may be noted that although the well-established correlation between CS and HAD (Staudte & Pette 1977, Bylund et al 1976, Örlander et al 1977) was found also in the present study no significant changes in HAD activity were observed.

It seems clear that the adaptive response in skeletal muscle is related to the work performed during training. Exercise of low intensity or short

daily duration has been shown not to influence metabolic capacity of rat hindleg muscles (He, Wamio 1956, Holloszy 1967, Saubert et al 1975, Benzi et al 1975, Åström, personal communication). With an increased training intensity and duration a progressive increase in mitochondrial enzyme activities towards a new steady state takes place (Kraus et al 1969, Barnard & 1971, Guy & Snow 1977, Henriksson & 1977, Baldwin et al 1977). It appears that the intensity is of major importance when the duration is relatively short (Benzi et al 1975). At high intensity the duration per workout is the determinant for the training response (Fiebert 1975, Dohm et al 1977). Ultimately a training level is reached beyond which further prolongation of workout is without effect on muscle metabolism (Terjung 1976, Vihko et al 1978).

The lack of effect on muscle metabolism in the present study is in contrast to our previous findings (Örlander et al 1977). The reason for this is probably the differences in the employed training programs. About one third of the training volume in the previous study consisted of calisthenics with aim to increase muscle power whereas in the present study training was carried out in the present study. Bylund et al (1977) using a training program involving leg strength training found that the increase in oxidative capacity was largely confined to type II fibres. Since endurance running (and probably also walking) is performed mainly with the aid of type I fibres (Costill et al 1973) it is reasonable to conclude that low intensity exercise within the capacity of this fibre population at the work load involving also the type II fibres then be required to elicit an adaptive response.

In conclusion the present results show that maximal aerobic power can increase considerably without a corresponding change in muscular oxidative capacity. Thus leg muscle oxygen utilization capacity does not seem to limit the whole body  $\dot{V}_{O_2}$  max. This conclusion is in accordance with our previous observations on low intensity training (Örlander et al 1977) and with the results of recent studies on detraining showing a clear distinction between changes in  $\dot{V}_{O_2}$  max and muscle enzyme activities (Henriksson & Reitman 1971, Houston et al 1979). Further support for this conclusion comes from the numerous studies where arterial oxygen content has been manipulated during maximal work (e.g. Ekblom et al 1975, 1976).

from experiments on maximal work involving great amounts of the body's muscle mass (Bergh 1976). As Clausen (1977) has pointed out, it is clear that  $\dot{V}_{O_2}$  max during work with large muscle groups is limited by oxygen supply. It is never possible that an elevated oxidative capacity in leg muscles other than the vastus lateralis have made some contribution to the increase in  $\dot{V}_{O_2}$  max. It has been shown that the gastrocnemius and soleus are considerably more involved during level running, whereas the vastus lateralis is not equally active during uphill or downhill running (Costill et al 1974). To our knowledge, no data are available as to the relative involvement of different leg muscles in the kind of walking and jogging activities employed in the present study.

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## Comparison of mucus collecting methods in fish olfaction

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A new method for collecting non-contaminated skin mucus from fishes is introduced. The method is compared with the customary method by gel filtration, analysis of free amino acids, and determination of dry weight. Skin mucus collected by the customary method is shown to be contaminated by intestinal juice. The role of skin mucus as a source of fish odour affecting fish behaviour is discussed in the light of the above findings.

**Key words:** Amino acids, fish odour mucus, olfaction, salmonids, fish behaviour.

Our work with olfaction in fishes (Wrede (1932)) showed that the skin mucus from the minnow (*Osmerus laevis* Ag.) contained an attractive substance. Todd et al. (1967) gave evidence that the low bullhead (*Ictalurus nebulosus*) recognize individuals of their own species from substances present in the skin mucus by sense of smell. Skin mucus has also been thought to be the source of specific odorous substances in the homing and zonation of anadromous salmonids (Nordeng 1977). This last suggestion has been supported by electrophysiological studies by Døving et al. (1973, 1974) on the olfactory sense of the arctic char (*Salvelinus alpinus* L.). Potent olfactory substances seem to present also in the skin mucus of other teleost species (*Salmo gairdneri*, *Coregonus clupeaformis* and *Carassius auratus*) as demonstrated by Hara & McDowall (1976).

The skin mucus substances of some fishes were studied by Wersler & Wersler (1957). A protein said to contain 16 amino acid residues was found to be the predominant component. Enomoto et al. (1961) found only small variations in amino acid composition of skin mucus proteins in 11 species examined. The skin mucus from Atlantic salmon (Harris & Jørgensen (1973)) established the presence of 18 amino acid residues in dry weight samples of 62% protein content and an analysis of glycoproteins on skin mucus from the char by Wold & Selset (1976) re-

vealed 17 common amino acid residues. No investigator has analyzed the content of free amino acids in the skin mucus which olfactory-wise would give the most valuable information. The olfactory stimulating properties of amino acids were well demonstrated by Sutterlin & Sutterlin (1971), Suzuki & Tucker (1971), Hara (1973) and Belghazi & Døving (1977). Recent findings by Thommesen (1978) of a spatial distribution of odour induced potentials in the olfactory bulb in salmonids, however, indicates that a distinction must be made between amino acids and other stimulating substances.

Working with skin mucus in the field of olfaction, Selset & Døving (1980) focused attention also to intestinal contents in behavioral experiments with arctic char (*Salvelinus alpinus* L.). Since an extract of food pellets seemed nonattractant, the attraction to food might be excluded. Our attention was then focused on the manner by which surface mucus was collected. Uskova et al. (1971) had shown that the utmost care has to be taken in the collection of skin mucus. Their blowing off of the mucus with a jet of air, or scraping with a scalpel gave 9 amino acid residues in the hydrolyzed mucus proteins contrary to the usual 16. Microscopic examination showed that the 'customary' method gave contamination of the mucus by epithelial cells. Together with the above findings, this gave rise to the following ques-

Table 1 Percentage composition of low and high molecular weight material and contents of free acids in pure mucus, customary collected mucus and intestinal contents from char based on weight

Material	Contents of low m.wt material (%)	Contents of high m.wt material (%)	Contents of free amino acids (%)
Pure mucus	41	59	15.4
Customary collected mucus	73	27	10.3
Intestinal contents	90	10	—

tion. Could the customary method for collection of skin mucus also result in contamination by intestinal contents?

In the present study we give evidence that skin mucus collected in the customary way is indeed contaminated by intestinal contents. An improved method for collecting skin mucus from fishes is introduced, and the amount of free amino acids in this mucus is measured.

## MATERIAL AND METHODS

The skin mucus was collected from Atlantic salmon (*Salmo salar* L.) and arctic char (*Salmo alpinus* L.). All fishes were artificially hatched and raised at the Research Station for Salmonids, Surnadalssøra, Unit Norway.

**A customary method for collecting mucus.** A fish is immobilized by a stroke on the head above and behind the eyes. Bleeding is avoided by using a rounded wooden club. The fish is carefully held with two fingers behind the operculum and the mucus gently scraped off from top to bottom with a knife. The scraping might be repeated after moistening the skin surface with distilled water. Squeezing out feces was avoided, although small amounts of light yellow juice could sometimes be observed draining from the anal opening. This method for collecting skin mucus is called the "customary way" in the following text, i.e. according to Uskova et al. (1971).

**The developed method.** After immobilizing the fish it was hung from the mouth cartilage with fish hooks. The skin surface was washed with a spray of distilled water. The skin surface was then "vacuum-cleaned" with an aspirator system connected to a collecting flask. The mouthpiece of the vacuum-cleaner was flattened at the end and gently rounded to avoid sharp edges. Skin mucus was gently collected from the area anterior to and above the gut opening. Since the skin mucus of a swimming fish may contain substances from the surrounding water, the mucus collected by the developed method is not pure in the chemical sense of the word. In the following context, however, "pure" skin mucus is used as a synonym for "vacuum-cleaned" skin mucus. Intestinal contents were

removed from the same fishes as were vacuum-cleaned. Since fish food rests had been shown to be of no importance for our work (see Introductory part), the gross mucus feces was eliminated by fasting the fishes for 48 h before the material was collected by our developed method. The intestine was cut posterior to the p. caeca and close to the gut opening. A small amount of distilled water was introduced into the closed intestine and after some minutes the mucus content was squeezed out.

**Examination of the material.** Acetic acid was added to pure mucus, intestinal contents, and customary collected mucus to a final concentration of 5%. This procedure precipitates the high molecular weight material, which might be used as an alternative method to achieve isolation of fish odorants (Selset 1980). The mixture was centrifuged at 25 000  $\times g$  and the UV absorption spectra of the supernatants were taken in the area 400–600 nm. After lyophilization the dry weight was measured for both supernatant and precipitate of pure mucus and intestinal contents, together with the dialysate and residual customary collected mucus. The supernatants from pure mucus (2), intestinal contents (3) and customary collected mucus were chromatographed on Sephadex G-25 column (Elutiron with 5% acetic acid) and material with absorbance at 280 nm.

2 ml mucus collected from the skin by the vacuum cleaning method was transferred directly into a solution of 1 ml 10% TCA. The content of free amino acids was then analyzed on a Bio Cal BC 700 Amino Acid Analyzer, using the regular protein hydrolysis program for one column. Mucus collected by the customary way was first dialyzed with a cut-off at approximately 12 000 Daltons and the dialysate was analyzed for its content of free amino acids. The dialysis buffer used was from A. H. Thomas Co. (Cat. No. 443-100).

## RESULTS

The dry weight contents of low molecular weight material in the pure mucus has a lower value compared to the high molecular weight material (Table 1). In the material testing

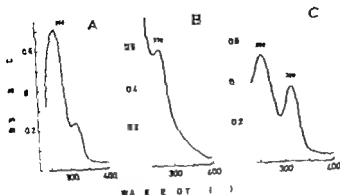


Fig. 1 UV-absorption spectra, 240–400 nm, for the low molecular weight material from Atlantic salmon. (A) Pure skin mucus, (B) intestinal contents, (C) Customary collected skin mucus.

low molecular weight material predominates. Comparison of low- and high-molecular weight material of customary collected mucus shows intermediate values between pure mucus and intestinal contents. The values obtained for the customary collected mucus are not directly comparable to those for pure mucus and intestinal contents since two different methods for fractionation were used. However, if precipitation had been used for the customary collected mucus, the portion of molecular weight material would have been different, still higher than the value obtained since dialysis contained no acetic acid precipitable material.

The UV absorbance spectra of the low molecular weight material are presented in Fig. 1. The absorbance spectrum of the supernatant from customary mucus (Fig. 1C) differs from that of pure mucus (Fig. 1A) with respect to  $\lambda_{max}$  at 260 nm and  $\lambda_{min}$  relative to  $\lambda_{max}$  at 268 nm and 318 nm. This shift might be due to contamination by epithelial cells as postulated by Unkova *et al.* (1971). The supernatant from intestinal contents has only one peak at 272 nm (Fig. 1B).

Size exclusion chromatography of the three types of supernatant are presented in Fig. 2. In an ordinary gel filtration, substances are eluted between the void volume ( $V_0$ ) and the salt volume ( $V_s$ ). The gel filtration of the material in this work produces peaks after the salt volume, a retarding effect known possibly to be due to aromatic compounds (Anonymous 1974). The gel filtration of the supernatant from pure mucus gives 3 peaks after the salt volume (Fig. 2A) indicated with Roman numerals in the figure. The supernatant from intestinal content, however,

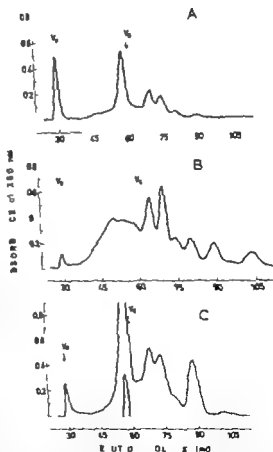


Fig. 2 Elution diagrams from the Sephadex G-25 chromatography of the low molecular weight material from Atlantic salmon formed after precipitation with acetic acid. (A) Pure skin mucus, (B) intestinal contents, (C) Customary collected skin mucus.  $V_0$  = void volume,  $V_s$  = salt volume. Special retarded peaks are marked with Roman numerals. Note that the elution speed in the three diagrams is different.

Table 2 Concentrations of free amino acids found in pure skin mucus from char

Amino acid	Conc (nmol/ml)	Amino acid	Conc (nmol/ml)
Asp	179.8	Met	23.6
Thr*+Ser*+		Ile	40.1
Asn+Gln	49.7*	Leu	73.5
Glu	145.8	Tyr	138.0*
Pro	Trace	Phe	154.5*
Gly	75.2	Lys	51.1
Ala	103.4	Arg	31.4
iCys	Trace	His	98.8*
Val	100.8		

Amino acids found by Uskova & Chaykovskaya (1971)

Insufficient separation for individual quantification (See examination of material)

\* Possible disturbance from hexoseamines. Perhaps also methylhistidine

shows 8 peaks after the salt volume (Fig. 2B). The same 6 peaks are also found in the gel filtration curve from a supernatant of "customary" collected mucus (Fig. 2C). This result suggests that the "customary" collected skin mucus is contaminated with intestinal juice. The dry weight values for customary collected mucus (Table 1) are more like those obtained for intestinal contents than for pure mucus. Since the relative volume of intestinal contents in customary collected mucus can be assumed small, the result can only be explained if the total dry weight per volume of intestinal contents is considerably higher than that of pure mucus.

The amino acid analysis revealed 19 different common free amino acids in "pure" mucus. The data also suggest a considerable amount of taurine and maybe also of phosphoserine. The characteristic peaks for the two substances fuse in the elution program used making discrimination difficult. Threonine, serine, asparagine and glutamine are insufficiently separated and cannot be quantified individually. In addition, tryptophane may also be present but could not be measured in the system used. The identified amino acids are listed in Table 2 together with their individual concentrations.

On a dry weight basis the amount of free amino acids in "pure" skin mucus is higher than in customary collected mucus (Table 1). The content of amino acids in intestinal contents was not measured. However, it may be assumed very low because of both intestinal and bacterial absorption.

## DISCUSSION

The gel filtration of this work shows that the intestinal contents (Fig. 2B) contain substances which give a retarded peak no. VI not found in the pure skin mucus (Fig. 2A) collected by the mixed method. Since skin mucus collected in the customary way also shows a similar retarded peak, this points to a contamination from internally. The UV absorbance spectra indicate a contribution also from other sources. Dead and healthy epithelial cells naturally make a contribution.

Pure mucus (Peak no. VI) however shows its absence in the gel filtration of pure mucus from all fasted groups examined. This points to the conclusion that the origin of peak no. VI in the customary collected mucus is not the epithelial cells. The fasting of the fishes before the collection of material by the new method also prevents uncertainty compared to the customary collection of mucus from normal fed donors. The different handling might give a possible explanation of variation in magnitude of the gel filtration peaks between the collection methods used. The latter does not however explain the total absence of peak no. VI in "pure" mucus, since this peak is found in the intestinal contents from the same fishes. Concerning olfaction, the present work therefore indicates that the utmost care has to be taken in the collection of odourant material from fishes. Contamination of the type shown are likely to be even more detectable by the fish olfactory organ than by molecular methods. Working with skin mucus as a source of olfactory material, the demonstrated method of collection seems at present to be the best.

The present amino acid analysis of the skin mucus collected by the "vacuum-cleaned" method reveals that nearly all the common amino acids found as such by Uskova & Chaykovskaya (1971) found by thin layer chromatography, only 10 amino acid residues in the hydrolyzed skin mucus protein, but thin layer chromatography is not as sensitive as a modern amino acid analyzer. In addition, the free amino acids do not necessarily separate from the mucus proteins, since epithelial cells and hemolyze at the tissue surface. Anyhow, the highest concentration of amino acids revealed in our experiments are mainly in accordance with results of Uskova & Chaykovskaya; the amino acids found in their experiment are marked with asterisk in Table 1.

was established through the above experiment to give a background for the following question: skin mucus be source of olfactory substances to fishes. To answer this question, a brief must be given of skin mucus sampling methods used in prior reports.

ode (1934) collected the mucus for her work by using the fishes in a net above water for some and then sampling the mucus from the net leag (1971) and Døving et al. (1973, 1974) collected the mucus by carefully rubbing anaesthetized nethy-killed fishes followed by washing with water. H. Nordeng, K. M. Døving, Personal communication). Hara & MacDonald (1976) washed the surface of the fish with a water jet. All these sampling methods may give contamination by urine-jaccet. Todd et al. (1967) collected the mucus their work by wiping the dorsal surface of the with a piece of wet cloth. This method for plucking skin mucus seems fairly good and the it seems to establish reliable evidence for the existence of specific odorous substances in the mucus at least for the particular species used. ever the authors remarked that the responses of the sensitive in neutral water enriched by the or alone than to the donor water itself. Lack of residues or mine to the mucus-enriched water proposed as an explanation for the lower re-

action et al. (1973) have reported that certain low molecular substances (less than 1000 Daltons) from horse stream water act as olfactory stimulants derivatives (Alosa pseudoharengus). The absence detectable amount of amino acids in the water indicated that free amino acids were not involved in horsestream response.

The present experiment establishes the presence of free amino acid in the skin mucus of fishes. ce the olfactory stimulating properties of amino acids are demonstrated there can therefore be doubt that the skin mucus is source of olfactory stimulating substances. It is difficult however to argue that amino acids have any signal properties for food detection. The specific olfactory stimulating substances which have been demonstrated by some of the authors mentioned may have their origin in the skin mucus. We are not to see however that any definite proof has been given for the skin mucus alone acting as a source of such substances. The present work suggests further investigation in this field.

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Table 2 Concentrations of free amino acids found in pure skin mucus from char

Amino acid	Conc (nmol/ml)	Amino acid	Conc (nmol/ml)
Asp	129.8	Met	25.6
Thr*+Ser*+		Ile	40.1
Asn+Gln	249.2*	Leu	73.5
Glu	145.8	Tyr	138.0*
Pro	Trace	Phe	154.5*
Gly	75	Lys	51.1
Ala	103.4	Arg	31.4
$\gamma$ -Cys	Trace	His	98.8*
Val	100.8		

Amino acids found by Uskova & Chaykovskaya (1971). Insufficient separation for individual quantification (See examination of material).

\* Possible disturbance from hexoseamines. Perhaps also methylothymidine.

shows 6 peaks after the salt volume (Fig. 2B). The same 6 peaks are also found in the gel filtration curve from a supernatant of "customary" collected mucus (Fig. 2C). This result suggests that the "customary" collected skin mucus is contaminated with intestinal juice. The dry weight values for "customary" collected mucus (Table 1) are more like those obtained for intestinal contents than for "pure" mucus. Since the relative volume of intestinal contents in "customary" collected mucus can be assumed small, the result can only be explained if the total dry weight per volume of intestinal contents is considerably higher than that of "pure" mucus.

The amino acid analysis revealed 19 different common free amino acids in "pure" mucus. The data also suggest a considerable amount of taurine and maybe also of phosphoserine. The characteristic peaks for the two substances fuse in the elution program used making discrimination difficult. Threonine, serine, asparagine and glutamine are insufficiently separated and cannot be quantified individually. In addition, tryptophane may also be present but could not be measured in the system used. The identified amino acids are listed in Table 2 together with their individual concentration.

On a dry weight basis the amount of free amino acids in "pure" skin mucus is higher than in "customary" collected mucus (Table 1). The content of amino acids in intestinal contents was not measured. However, it may be assumed very low because of both intestinal and bacterial absorption.

## DISCUSSION

The gel filtration of this work shows that the tinnal contents (Fig. 2B) contain substances giving a retarded peak no. VI not found in the skin mucus (Fig. 2A) collected by the usual method. Since skin mucus collected in the customary way also shows a similar retarded peak, this points to a contamination from intestinal juice. The UV absorbance spectra indicate a contribution also from other sources. Dead and leaved epithelial cells naturally make a contribution to "pure" mucus. Peak no. VI however shows absence in the gel filtration of pure mucus from all fasted groups examined. This points to the conclusion that the origin of peak no. VI in the customary collected mucus is not the epithelium. The fasting of the fishes before the collection of material by the new method also prevents contamination compared to the "customary" collection of mucus from normal fed donors. This difference in handling might give a possible explanation of variation in magnitude of the gel filtration between the collection methods used. The result does not however explain the total absence of peak no. VI in "pure" mucus since this peak is found in the intestinal contents from the same fishes. Concerning olfaction, the present work therefore shows that the utmost care has to be taken in the collection of odourant material from fishes. Contaminations of the type shown are likely to be even more detectable by the fish olfactory organ than by most chemical methods. Working with skin mucus as a source of olfactory material, the demonstrated method of collection seems at present to be the best.

The present amino acid analysis of the mucus collected by the "vacuum-cleaned" method reveals that nearly all the common amino acids found as such by Uskova & Chaykovskaya (1971) are found by thin layer chromatography, only 10% acid residues in the hydrolyzed skin mucus proteins, but thin layer chromatography is not as sensitive as a modern amino acid analyzer. In addition, the free amino acids do not necessarily originate from the mucus proteins since epithelial cells and hemolysate at the tissue surface. Anyhow, the highest concentration of amino acids revealed in our experiments are mainly in accordance with results of Uskova & Chaykovskaya; the amino acids found in their experiments are marked with an asterisk in Table 1.

# Chemical methods for fractionation of odorants produced by char smolts and tentative suggestions for pheromone origins

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Chemical procedures for fractionation of fish material containing odorants are described. These methods were developed with the aim of retaining the odorous activity of the material. Some of these methods were used to fractionate the intestinal contents of 1½ year old smolts of a population of anadromous char (*Salmo alpinus* L.). The fractions were prepared (a) for behavioural experiments to study possible attraction effects on mature char of the same population during the migration season, and (b) for testing in physiological experiments to elucidate the olfactory potency of the different fractions. The nature and origin of the attractive substances are discussed.

**Key words:** Fish, salmonid smolt, intestinal contents, chemical fractionation, odorants, pheromone, bile acids, macro organisms.

Anadromous salmonid fishes migrate from their living areas in the river systems out to sea and return to the same spawning sites as used by their parents (Clayton & Fries 1959). It has been shown that the olfactory sense is necessary for their correct return (Wisby & Hasler 1954, Groves et al. 1964, Hasler 1968 and Hasler et al. 1978). It is assumed that correct return is based upon prior learning to substances from plants and minerals characteristic of the home stream water. Nordeng (1957) has proposed a pheromone hypothesis for migration of the anadromous salmonids, char (*Salmo alpinus* L.), trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar* L.). According to this hypothesis, the homeward migration is an innate response to trouts of population specific substances released from the fry and descending in the same population. According to the hypothesis of Nordeng, mature char should be attracted to odorants produced by trouts of the same population during the season for spawning and migration. It should therefore be possible to isolate such substances and to establish their attraction effects and olfactory potency. In the present study different chemical methods are de-

scribed which were found suitable for fractionation of the biological material from smolts for subsequent testing in behavioural and physiological experiments.

## MATERIAL AND METHODS

**Material.** The material used in the present study was collected from 1½ year old smolts of a population of anadromous char (*Salmo alpinus* L.) hatched and reared at the Fish Breeding Experimental Station at Suzundshøra in Western Norway but originating from Lake Storsvatnet near the city of Hammerfest in Northern Norway (70°40'N, 24°02'E). All available information evidences the existence of only one population of anadromous char in this lake (Nordeng, pers. comm.). During breeding at the Fish Breeding Experimental Station the population was kept genetically closed.

**Handling of the material.** Since the olfactory system of the char is highly sensitive and since the postulated pheromones are expected to be present in minute quantities, great care was taken to avoid contamination of the material. Therefore no solvent, reagent or other material that would add odorants to the biological material or disturb the olfactory system of the fish, was used unless it could be removed completely after use. To fulfill these requirements all solvents and material that were brought into contact with the odorants were tested on the olfactory



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Table 1. Final results of the behaviour tests on mature Hammerfest sea-char for attraction to material released from smelts of the same population. Probability values are correct only if the binomial model is valid for the experiments.

solvent	Total no. of trials (N)	No. of successful trials (X)	Probability in % for X or more successful trials of N trials
interface sea-char smelt	313	19	<0.01
interface sea-char smelt, visual contacts (HA)	761	165	0.01
interface sea-char smelt, station	183	90	36
	18	123	3.4
	105	55	35
	150	79	28
	210	119	9.3
	71	25	96 <sup>a</sup>
A (1 through 4)	123	66	3
B-1	42	13	94 <sup>a</sup>
B-2	41	23	50
B-3	114	68	2.4
B-4	51	29	20
B-5	33	16	50
B-6	31	10	96

# relative effect of the fraction

at 40°C to remove acetic acid and was then stored at -20°C. The other portion was fractionated further according to the precipitation method. Analysis by using 100 ml of 1 M HCl (pH 1.0) was used. The solution was then stored at -20°C. The solution was then stored at -20°C. The solution was then stored at -20°C.

and G-25 chromatography. The supernatant A) was chromatographed on 10 ml portions on G-25 (Bio) column (Pharmacia Fine Chemicals) at 1 ml elution speed 110 ml/h. fraction volume 0.3 ml. eluting with H<sub>2</sub>O in air. The elution was monitored by Bio-Rad Model 1300 UV detector at 254 nm. The collecting tubes are pooled along to the peak in the elution diagram and acetic acid removed as described above. Fractions were stored at -20°C.

reverse chromatography on Amberlite XAD-2. The material of the fraction called HAS- (Fig. 2) was eluted with 1 ml of water and 9 ml of 0.1 M NaOH (Nakano & Sjogvall 1977). The solution was chromatographed on an Amberlite XAD-2 column (R.D. 44 1 cm, elution speed 3 ml/min) at 4°C. The column was pre-equilibrated with 10 ml of methanol, water and acetic acid. Substances not adsorbed (HAS- A) were eluted with 100 ml of water and taken to dry by rotary evaporation. NaOH was not removed. Material (HAS- B) was eluted with 100 ml of

methanol, taken to dryness as above and stored at -20°C.

Sephadex LH-20 chromatography. HAS-2-A and HAS-2-B were dissolved each in 1 ml of methanol for chromatography on a Sephadex LH-20 column (70 1 cm, elution speed: 17 ml/h, fraction volume: 2 ml) and eluted with methanol at 4°C. Most of the NaOH in HAS-2-A was not dissolved in methanol and was centrifuged off. The elution progress was monitored in the same way as above. The collecting tubes were pooled as indicated in the elution diagram (Fig. 3), and the fractions were taken to dryness and stored at -20°C.

Thin layer chromatography. 10 10 cm thin layer silica plates (DC-Alufolien Kieselgel 60 F 254 R<sub>10</sub>, Merck Art. 3334) were used with the solvent system chloroform/methanol/25% NH<sub>3</sub> in the volume ratio 2/2/1. The solvent front was allowed to reach the edge of the plates. Substances were visualized by means of an ultraviolet lamp (254 and 366 nm) normally in dark spots on a fluorescing background using 254 nm UV-light. However, some of the substances were themselves fluorescing when UV light of 366 nm was used.

Dry weight measurements. Aliquots of the different fractions and the starting material were taken out for lyophilization and weighing to determine the dry weight of the material.

Gas-liquid chromatography-mass spectrometry. To identify possible bile acids in the fraction HAS-2-B 0.2 mg of this material was subjected to permethylation and subsequent analysis by gas-liquid chromatography-mass spectrometry as described by Alen et al. (1977). The analyses were carried out at Karolinska Institute Stockholm by J. Sjogvall in January 1979.

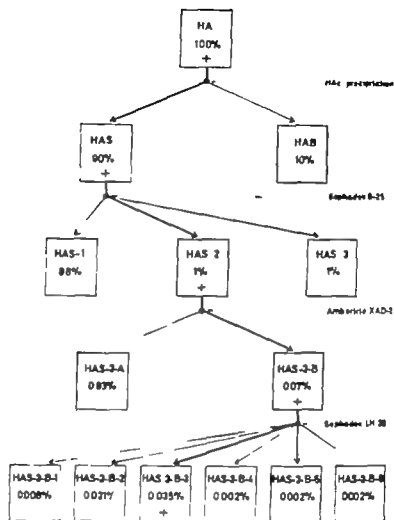


Fig. 1. The main lines of the isolation procedure. Proportions of dry weight material are given in per cent. Attractive fractions are marked +.

system of char with electro-physiological method as described by Thommesen (1978). All glass equipment were washed with chromic sulphuric acid water, 0.5 N hydrochloric acid water and then dried at 140°C. All equipment to be used for the odorant was handled with Triflex gloves (Code FND 7073, Triflex Laboratories Ltd.) Likewise tested by electro-physiological method. All water used was double distilled with a Quickfit 3DWS Double Distillation Water Still (Coming Ltd.). It was found essential that the double distilled water be tested with regard to olfactory potency at regular intervals in case of any microbial growth. This is a problem especially when polyethylene storage containers are used.

**Collection of material.** The skin mucus of molt has been thought to be the source of the postulated migration pheromones in salmonids as reported by Nordeng (1971) and Døving et al. (1973). However, behavioural experiments (Sæset & Døving 1980) on anadromous char with pure skin mucus collected with new method (Ståbø & Sæset 1980), and intestinal content revealed that only the latter was a source of substances attracting mature char during the migration season. The chemical study was therefore focused on the intestinal content of char

molt. The large intestine from the pharynx, caudal gut opening of 30 newly killed fishes were cut out and content (0.1–1.0 ml per fish) squeezed directly in 1 ml centrifuge tubes. The volume of each tube adjusted to 8 ml by addition of water. The tubes were vigorously and particles were centrifuged off at 14,000 g in Sorvall Superspeed RC-8B automatically refrigerated centrifuge for 30 min at 0°C. The supernatant pooled and used as starting material for the experiment.

**Precipitation of high molecular material.** Acetic acid was added to the pooled supernatant (HA) to a final concentration of 5%. By comparison found that all the high molecular weight material that was retained in dialysis bags with a molecular cut-off of approximately 1,000 Dalton and for some other substance a well precipitated method. The precipitate was centrifuged off and dialysed and washed with about 30 volumes of 0.1 M acetic acid 3 times and once with water. The precipitate was lyophilized and stored at -20°C until the supernatant HA was dialysed into two portions. Portion was taken 1 dryness 3 times with distilled water in a rotary evaporator.

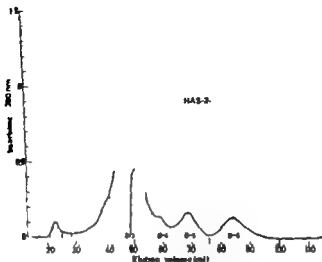


Fig. 3. Elution diagram for Sephadex LH 20 chromatography of the fraction HAS-2-B. The upper part of the peak HAS-2-B is inserted in its base (max. abs. 3.0).

char seemed to behave indifferent towards the fractions.

The chromatographic procedures used in this work seem to give only small loss of material. The losses could only be estimated because some of the fractions were difficult to dry.

The fraction HAS-2-B-3 was found in the biological tests, to have a threshold value for olfactory response that was a 100 times lower than other fractions. In fact the odorants in this fraction seemed to be as potent as bile acid derivatives which are the most powerful odorants found in fish so far (Doving, Selset & Thomassen 1971).

The results of some of the thin layer chromatograms of the different fractions are seen in Fig. 4. In this figure are shown the spot patterns found by elution of HAS-2, the XAD-2 fractions A and B and the subfractions of HAS-2-B from the LH-20 column. The HAS-2-B-3 chromatogram shows six spots of which three are fluorescent at 366 nm. Five of these spots may be due to substances also present in neighboring fractions. The nature of the spots in this chromatogram suggests that HAS-2 contains mainly 3 different substances, the non-fluorescing spots. The gas-liquid chromatography—

mass spectrometry analyses showed with a high degree of reliability that cholic acid is the main component of HAS-2-B-3. Cholic acid has an  $R_f$  of 0.5 in the TLC system used that coincides with

the second nonfluorescing spot of HAS-2-B-3. A second main component was believed to be some kind of branched hydrocarbon which was not further identified. A third substance was tentatively identified as dioctylphthalate (plasticizer) a normal contaminant of such systems. These three substances are believed to be the cause of the three nonfluorescent spots in the chromatogram of the fraction HAS-2-B-3. No further investigations into the structure of the fluorescent minor components of this fraction have been carried out so far.

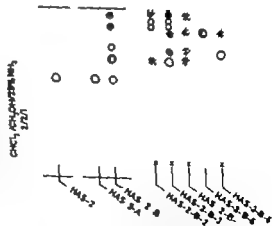


Fig. 4. Thin layer chromatograms of some of the fractions obtained. Dark spots (see text) are marked with open circles. Fluorescent spots are hatched.

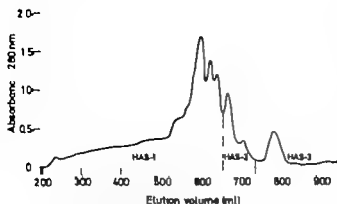


Fig. 2 Elution diagram for Sephadex G-25 chromatography of the supernatant HAS. The salt volume was 565 ml.

## RESULTS

The outlines for chemical fractionation used in the present study were governed by results from the behavioural studies. These studies were made during the period of homeward migration normal to mature char of the wild population. These results are described in a subsequent article (Selset & Døving 1980). Additional information for the chemical work was obtained by studying the physiological effects of the chemical fractions on the olfactory organ of the char.

The diagram in Fig. 1 gives the main lines of the isolation procedure used in the present study. High molecular components of the intestinal contents were precipitated. The attractive supernatant was first fractionated on Sephadex G 25 and further by the use of Amberlite XAD-2. Finally the material was fractionated with a Sephadex LH 20 column. The fraction HAS 2-B 3 most likely contained the attractive substances. This fraction contained 0.035% of the water soluble part of the intestinal contents. The initial fractionation of the intestinal contents (HA) by precipitation probably results in a precipitate contaminated with low molecular material because of adsorption effects, but this does not include the substances attracting mature char as determined by behavioural experiments. The attractants seem to be present only in the supernatant (HAS).

The final results of the behavioural experiments (Selset & Døving 1980) with their respective probability values calculated using the binomial model are presented in Table 1. These data are presented more comprehensively in the referred article.

The supernatant was fractionated further on a

Sephadex G 25 column. The elution diagram (Fig. 2) shows an elution process that deviates from ordinary gel filtration in that some of the material is strongly retarded. Especially aromatic substances are known to be retarded in this way (Sorenson 1967).

Originally the eluate from the G-25 column was divided into 7 fractions according to the gel diagram, but as the behavioural experiments established the presence of attractants only in fraction called HAS-2 in Fig. 1 the other fractions were pooled as shown in the figure. The bulk of amino acids were eluted just before the salt ( $V_{NaCl} = 565$  ml). The attractive fraction HAS-1 contained only 1% of the starting material (HA) by weight. HAS-2 is one of the retarded fractions in a concentration of 1 g/l in water it also forms micelles that are clearly visible on cooling the solution. This means that HAS-2 could contain lipids that also might possibly be of aromatic character. This fraction has a relatively high absorption of ultraviolet light in the range of 280 nm. The electro-physiological responses recorded from the olfactory bulb induced by HAS-2 had similar properties to those obtained by stimulation with bile acids and derivatives (Døving, Selset & Thommesen 1980). These facts indicated that attractants could be some kind of bile acid derivative. For this reason the next fractionation was chosen to be adsorption chromatography on a column of Amberlite XAD-2, a resin known to adsorb amongst other compounds bile acids. More than 90% of the HAS-2 contents (called HAS-2-B) passed right through the column while the (HAS-2-A) was adsorbed to the resin. Whether or not methanol will release completely all the substances adsorbed is not known, but the attractants seem to be eluted by this solvent.

Further fractionation of HAS-2-A and HAS-2-B was accomplished by use of the lipophilic Sephadex LH 20 with methanol as eluent. Fairly good fractionation was achieved of the HAS-2-B contents (HAS-2-B 3) whereas the HAS-2-A substances were not separated well by this method. The fraction HAS-2-B 3 contained the rest of the NaOH originally added and no other substances as judged by thin layer chromatography and pH tests, and therefore NaOH presented no problem to the physiological and behavioural experiments. According to the behavioural experiments the fraction HAS-2-B 3 should contain the attractants.

er and secreted into the bile but it seems dubious then to transport them the long way through the intestine where they will be exposed to bacterial attacks even though the intestinal microorganisms seem to be much less numerous in fish than they are in mammals (Sacquet et al 1976). The exact identification of the structures and of these substances require further investigation.

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## DISCUSSION

The present study gives examples of chemical methods we believe effective in handling odorant containing material from fishes in such a way that the biological activities of the odorants are likely to be retained. The methods used are also suitable for physiological tests on the olfactory organ. The main topics discussed are the origin of the attractants, the precautions necessary for their biological effects to be retained, the chemical nature of the attractive substances, and the consequences of the chemical nature of such substances in the life history of migratory salmonid fishes.

The material studied in the present investigation was intestinal contents of char smolts sampled at the beginning of June. This period was selected since it coincides with the season for homeward migration of mature char (Nordeng 1977). The smolts were from the in-bred population of chars raised at the Fish Breeding Experimental Station and not from the wild population. It is not known if the production of attractants are restricted to the migration season. It is also uncertain if the postulated pheromones will be produced at all in an artificially raised population. However we have shown that the smolts do produce substances that attract the mature chars during the migration season (Selset & Doving 1980).

In chemical work with such odorants it is of the utmost importance to avoid any sort of odour contamination since there is no way of knowing in advance if they will affect the behaviour of the fish. It should be mentioned here that extracts of human skin have been reported by Idler et al. (1956) to induce fright reactions in some salmonid fishes. Great care should be taken to prevent microbial growth. Bacteria will be present in the starting material (Yoshimizu et al. 1976) and may rapidly transform many of the interesting substances (Hill 1976) into derivatives not produced in the intestine under natural conditions. Certain bacteria encountered in this work have been shown to produce odorants inducing electro-physiological responses similar to those of fraction HAS-7 B 3 and bile acids, and with similar threshold values.

Precipitation of the high molecular weight components of HA did not result in a fractionation of the material into odorants and nonodorants, presumably because of adsorption effects as already mentioned. Bile acids and derivatives, mostly microbially transformed, are known to be present in

considerable amounts in the large intestine, potency as odorants (Doving, Selset & Thommesen 1980) and their detergent effects could explain precipitate being a definite although weak stimulant.

The chemical structure of the attractive substances can partly be deduced from the GC information. The attractive fraction was retained on the G 25 column, indicating aromatic components. At high concentrations the fraction forms micelles which indicates presence of polar groups. The attractive substances were adsorbed to 'NAD-2' resin. These facts make it likely that attractants are some sort of polar steroid molecules possibly containing aromatic rings.

The main component of HAS-7 B 3 was identified by Sjowall to have the same retention time as cholesterol and the same mass spectrum as cholic acid, i.e. indeed a polar steroid. Cholic acid is produced by all salmonids (and many other fishes as well) (Ili & Sidorov 1973) and can therefore not function as a population specific pheromone. However, cholic acid is shown to be a strong odorant for salmonids (Doving, Selset & Thommesen 1980) and possibly be part of a multipheromone system homing in salmonids.

The other main components of HAS-7 B 3, dioctylphthalate and the branched hydrocarbons, are not likely to be either attractants or odorants for salmonids.

The minor components of the fraction, fluorescent substances, have not yet been identified, but these could turn out to be polar aromatic steroids.

The bile acids, known to have important physiological roles as detergents in the digestion of fat and to facilitate the uptake of certain metals, most likely be adsorbed to organic matter and released at the spawning sites in the water stream. Rate of adsorption and release will then depend on physical factors like temperature, water flow, etc. and the stream's composition of organic matter and minerals.

There is a theoretical possibility that these substances can be retained at the spawning site in enough to secure homing for the fish population even if the fishes are absent for a period of time (e.g. Pink Salmon) (Harden Jones 1968).

The origin of the attractants is uncertain. They may be produced by microorganisms in the intestine from bile acids. They may also be produced

# don of $\alpha$ -MSH on the release of neurotransmitters in the retina

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It is now rapidly accumulating that there are several classes of neurons in the CNS that operate with peptides as neurotransmitters or neuromodulators, and there is a reasonable chance that  $\alpha$ -MSH may be such a peptide, mainly because it seems to be present in certain specific neurons (Déry & LeMoal 1978, Dubé et al 1978, Pelletier & LeMoal 1978, Jacobowitz & O'Donoghue 1978). There is as yet no clearly defined function for  $\alpha$ -MSH in man, but over the years, many behavioral and physiological effects of it have been described (Kaslow et al 1976, Tilders et al 1977) including the eye (Nauck 1951). In the course of a study of possible peptide neurotransmitters we have therefore tested the effect of  $\alpha$ -MSH on the release of retinal neurotransmitters and found profound effects which are reported here.

**Materials and Methods.** Eyes of pigmented rabbits weighing 1-2 kg were used. 10 or 20  $\mu$ Ci of labeled glycine, GABA, 5-hydroxytryptamine or aspartate were injected intravitreally and the animal was killed by an i.v. air injection 1 or 4 h later. The posterior half of the eye was everted and opened on a glass dome in a small thermostated ( $^{\circ}$ C) chamber (Bauer 1977) and drip superfused (1 ml/min) with a balanced salt solution according to Sandoz & Ames (1976) equilibrated with 5%  $\text{CO}_2$  in 95%  $\text{O}_2$ . Synthetic  $\alpha$ -MSH (CIBA, batch 61-8a) was applied dissolved in the same solution and always equilibrated with the gas mixture at least 20 min at  $37^{\circ}\text{C}$  before application. The radioactivity of the superfusate was monitored in 1 mm samples. It has previously been shown that under these circumstances most of the radioactivity of the retina represents the unchanged transmitter [ $^3\text{H}$ ]-glycine, [ $^3\text{H}$ ]-GABA or [ $^3\text{H}$ ]-aspartate in the respective cases (see Kramer 1971, Bauer & Ehinger 1978). There is also evidence that most of the [ $^3\text{H}$ ]-5-hydroxytryptamine taken up into the retina remains as such (Ehinger unpublished). Therefore, any increase in the release

of radioactivity most likely reflects a release of the unchanged transmitter, even if it may appear in the effluent as a metabolite. In particular, this is the case for GABA (Bauer 1978). This assertion was corroborated in a few studies on the release of radioactivity from rabbits injected intravitreally with [ $^3\text{H}$ ]-dopamine or [ $^3\text{H}$ ]-5-hydroxytryptamine and treated with the monoamine oxidase inhibitor Pargyline (50 mg/kg i.p. 4 h) before the start of the superfusion. The effects of  $\alpha$ -MSH were in these cases indistinguishable from that seen in animals not pretreated with Pargyline.

**Results and comments.**  $\alpha$ -MSH induced a striking increase in the release of radioactivity from retinas which 4 h previously had been given either [ $^3\text{H}$ ]-dopamine or [ $^3\text{H}$ ]-GABA. The concentrations needed for a small but still discernible response in a single experiment were about  $10^{-8}$  M. The effect increased with increasing doses. At  $0.6 \times 10^{-6}$  M  $\alpha$ -MSH had pronounced effects on retinas loaded with [ $^3\text{H}$ ]-GABA or [ $^3\text{H}$ ]-DA (Fig. 1a and b) but no significant effect on retinas given [ $^3\text{H}$ ]-glycine or [ $^3\text{H}$ ]-5-hydroxytryptamine.

It was previously shown that at short times (1-1 h) after the intraocular injection of [ $^3\text{H}$ ]-GABA, the localization of radioactivity is mainly in the glial cells (Ehinger 1977) whereas the localization is in neurons at 4 h. This can be used to test whether the source of increased efflux of radioactivity is mainly from nerve cells or glial cells.  $\alpha$ -MSH ( $0.6 \times 10^{-6}$  M) was found to have no effect when applied to retinas taken only 1 h after the injection of [ $^3\text{H}$ ]-GABA, and it is thus most likely the neurons which are the source for the increased efflux of radioactivity induced by  $\alpha$ -MSH.

It is noteworthy that the concentration of  $\alpha$ -MSH needed to get effects is as low as around  $10^{-8}$  M or less. The excitant amino acids, glutamic acid and aspartic acid, which rank as the strongest exciters, have discernible effects in our test system down to  $10^{-6}$  M (Bauer 1977).  $\alpha$ -MSH in our hands thus





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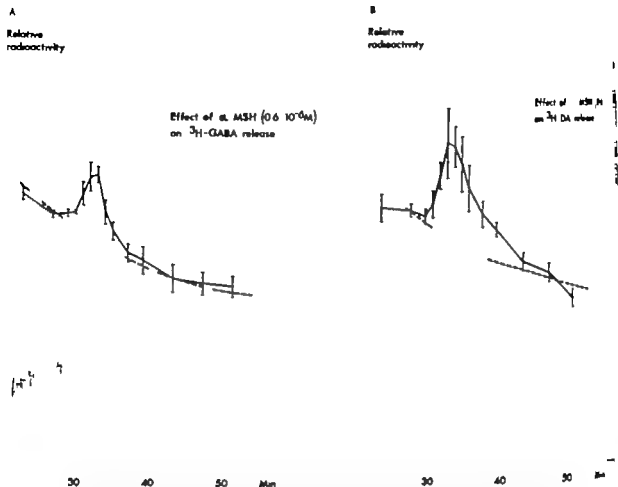


Fig. 1a. Effect in vitro of  $\alpha$ -MSH  $6 \times 10^{-6}$  M on the release of radioactivity from 4 rabbit retinas loaded in vivo with  $^3\text{H}$ -GABA intravitreally. Superfusion was started 4 h after the injection and the time given on the X-axis is after the start of the superfusion.  $\alpha$ -MSH was applied for 1 min as indicated. The individual release curves from 10 retinas were normalized as described previously (Bauer & Ehinger 1978) and the vertical bars indicate the standard error of the mean for the individual points. The normalization procedure transforms the radioactivity measurements into arbitrary relative units and there are therefore no scale markings on the y-axis. The unstimulated control efflux is represented by the dashed curve. The change in release rate induced by  $\alpha$ -MSH is significant ( $p < 0.001$ ) calculated as the difference of slopes of the curve segments between 30 and 33 min.

Fig. 1b. Experiment as in Fig. 1a but with  $^3\text{H}$ -dopamine labelling of retinal neurons. The change in release induced by  $\alpha$ -MSH is significant ( $p < 0.001$ ) calculated as the difference of slopes of the curve segments between 30 and 33 min.

ranks as the hitherto most powerful retinal exciter which raises the suspicion it is a neurotransmitter or a neuromodulator in the retina.

The site of action of  $\alpha$ -MSH is not known but the experiments permit certain conjectures. As noted above, it is likely to be on neurons rather than glia. Further, if its action were at the level of the photoreceptor synapses, it would seem likely to be detectable in all or most cells in the inner plexiform layer, i.e. it would be expected to be about the same on dopaminergic, GABA-ergic, glycinergic or indoleamine accumulating neurons which all are located in the inner nuclear and inner plexiform layers in the rabbit. Since the effect of  $\alpha$ -MSH is not equal

on these neuron types, it seems that  $\alpha$ -MSH acts at the second synapse in the inner plexiform layer. Here, dopaminergic neurons are contacted not only by amacrine cells (Dowling & Ehinger 1978) but also by the indoleamine accumulating ones, which are contacted almost exclusively by the bipolar cells (Ehinger & Holmgren 1979). We therefore guess that if  $\alpha$ -MSH is a neurotransmitter or neuromodulator in the retina, it is released by amacrine cells rather than by bipolar cells. However, before these conjectures can be validated or refuted more research is needed on the concentration and localization of  $\alpha$ -MSH in the retina and on its effects on individual retinal neurons.

# Induced epileptiform activity evoked from dendrites of hippocampal neurones

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Amplitude shift (DS) and spontaneous firing are known features of the epileptic neurone. DS is never evoked when the epileptogenic focus is applied close to the soma of neocortical neurones (Welsh 1971). This could be due to activity of the soma membrane to the epileptogenicity or to competitive effects evoked. As on single neurones have shown that epileptic mechanisms are referable both to dendritic and non-dendritic membrane properties. It is, however, not clear how dendritic properties are influenced in epileptogenesis. The hippocampal CA1 pyramidal cells, with separate populations on dendrites are useful when studying the epileptogenic role of dendrites. The present study is that the applied epileptogenic focus is restricted to the dendrites. A method of microinjection has recently been described by von Bartheld (1979). In this report that method is used to study the epileptogenic effects of penicillin applied to apical dendrites, resulting in action potentials recorded from the somata of pyramidal

neurons separately via platinum wire electrodes, placed among afferent parallel fibres impinging on corresponding dendrites. The afferent input was in many slices restricted to 80 µm wide bridges (Andersen et al 1979b). The stimulus strength was set at the threshold for action spike potential. An electrode for antidromic stimulation was placed upon the alveus. Intracellular recordings were obtained with glass electrodes placed under microscopic control in the soma layer. Current pulses were passed from the recording electrode. Conventional DC-recording of responses was made. The responses were photographed and analysed from film.

Records have been obtained from twelve CA1 cells. The resting membrane potentials ranged from -50 to -65 mV and the action potentials evoked were 70-95 mV in amplitude. The effects of apical dendritic microinjections were followed 5-40 min. Penicillin, ~0.5 M corresponding to 80-100 µm spherules injected distally among apical dendrites 300-400 µm from the cellbody rapidly causes depolarization, and firing of additional spikes. The depolarization appears as an afterdepolarization or as a DS during injection, or a few seconds later and remains for several minutes. Typically the changes relate to the synaptically evoked response from the injected apical dendrites. After injection the resting membrane potential remains virtually unaltered. The depolarization evoked in the cellbody by synaptically activated penicillin treated apical dendrites is enhanced and prolonged as compared to the generally unchanged basal dendritic response. The amplitude increase is of the order of 5-10 mV and the duration of the depolarization is prolonged by 10-50 ms. The first spike potential evoked by synaptic stimulation of penicillin influenced apical dendrites is identical with that of the control but elicited at slightly shorter latency and more regularly. Additional action potentials may ride on the

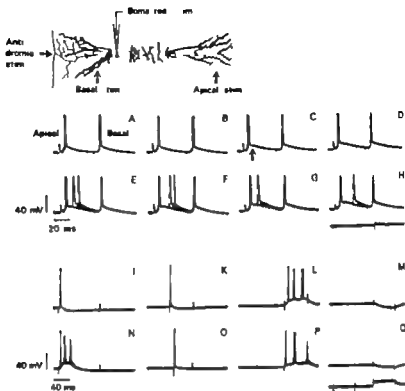
neocortical hippocampal slices were cut and maintained *in vitro* as described by Skrede & Westgaard (1979). Pressure microinjection of 0.3-1 µl 34 mmol penicillin, pH 7.3 was made from glass tubes with 2 or 5 µm tips. The 34 mmol penicillin chosen to achieve a rapid effect. The injections were made distally into the apical dendrites at a distance comparable to those of cell bodies recorded. Repetitive identical pressure pulses were given during 3-10 s time, depending on the amount injected. A study on <sup>14</sup>C-penicillin diffusion has shown the functional changes to be reported, referable to penicillin effects on apical dendrites. Synaptic activation of apical and basal dendrites, performed with 100 µs current pulses de-



neurons evoke epileptiform activity (Traub). Our observations are seen also when cells are loaded with weaker concentrations of  $^{14}\text{C}$ -penicillin, demonstrated to be confined to the apical dendrites. They are also in accord with those observed from CA 1 cells bathed in penicillin (Ander et al 1979; Schwartzkroin & Prince 1978).

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**Fig. 1** Inset: Diagram of experimental set up showing apical and basal dendritic and antidromic activation while recording from cellbody electrically also stimulated. Penicillin injection into apical dendrites (shaded). Intracellular records from two CA1 cells injected with penicillin. A–H and I–Q with 50 and 62 mV membrane potential. A: control synaptic activation via afferent bridges. 1st action potential evoked from apical dendrites. 100  $\mu$ A pulse action potential from basal dendrites. 5  $\mu$ A. B: 0.7 nl penicillin injected apically. record taken 2 s later. C: recordings 10 and 23 s after injection show DS and extra action potentials recorded 30 s. E: 41 s. F: 90 s. G, and 2 min after injection. Extracellular response shown below H. I–M: controls from the second cell. I: apical synaptic response to a 5  $\mu$ A pulse in a slice without bridges. K: Antidromic response to a 30  $\mu$ A pulse. L and M: soma responses to 1–0 nA pulses passed through the recording electrode. N–Q: corresponding records taken about 3 min after injection. Extracellular response shown below Q.

penicillin induced depolarization. Some are identical to the first one, others lower in amplitude. Spontaneous firing occurs at times. Samples of responses obtained are shown in the Fig. 1. The tracings A–H are from one, I–Q are from another cell. The first neurone was synaptically activated via 80  $\mu$ m wide afferent bridges placed near the apical (the first action potential) and basal dendritic segments (the late action potential). A is the control. B is taken 7 s after 0.7 nl penicillin has been injected. Note the change of the afterpotential. 10 s after start of injection (arrow in Fig. 1 C). Records D–H show further depolarization enhancement and additional action potentials evoked only from the now "epileptic" apical dendrites. The responses elicited from non-injected basal dendrites may at times show minor increase in depolarization, possibly due to the paired test situation. Antidromic invasion is usually not influenced. However, as shown in re-

cordings K and O from the second cell, antidromic responses obtained before and after injection may show slight changes in after-hyperpolarization. The synaptic response evoked via dendrites shows the typical depolarizing effect of penicillin (cf. I–N). Depolarizing pulses passed the recording electrode over the soma membrane showed in some cells a shortening of the latent period of the first action potential (cf. L and P). In other the opposite effect was seen. Increase in number of soma spikes was not seen. Hyperpolarizing pulses injected into somata of penicillin influenced showed no or minor alterations in electrical membrane properties (cf. M and Q).

The observations reported here illustrate that application of penicillin only to the dendrites of a neurone is sufficient to render the cell "epileptic" to synaptic input. The present findings fulfill the prediction from a neuronal model of altered dendritic properties (cf. M and Q).

# behaviour of mature anadromous char (*Salmo alpinus* L.) towards odorants produced by smolts of their own population

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SELSET J.F. & DØVING K.B. Behaviour of mature anadromous char (*Salmo alpinus* L.) towards odorants produced by smolts of their own population. *Acta Physiol Scand* 1980, 108, 113-122. Received 24 April 1979. ISSN 0001-6772. Institute of Zoophysiology, University of Oslo, Blindern, Norway.

Mature anadromous char (*Salmo alpinus* L.) of specific population were tested in behaviour experiments with respect to attraction effects of smolts of the same population, smolt material, and chemical fractions of that material. The char reaction indicated preference for intestinal contents and possibly bile from own population over skin mucus material and food. Chemical fractions isolated from the intestinal contents were tested for attraction at concentrations of  $1.5 \cdot 10^{-6}$  g per l. One of the fractions attracted the mature char. The results are discussed in relation to the migration pheromone hypothesis proposed by Nordeng (1971) as being crucial for the return of anadromous salmonids to their home river spawning grounds.

**Key words:** Fish salmonid char behaviour, smolt attractants, intestinal contents, bile, chemical fractions, migration, pheromone.

homeward migration of salmonids depend  
ly upon an intact olfactory organ as shown,  
by Håiby & Hæuser (1954), Groves et al. (1968),  
by Håiby (1975). These findings imply that the fishes are  
led by odour trails back to their spawning  
rivers. The substances responsible for these trails  
have been suggested to emanate from the plants and  
materials characteristic of the home stream water  
(Håiby 1966, Hæuser et al. 1978). Nordeng (1971,  
1977) has proposed that the substances are released  
by the smolts of the same populations as the  
spawning fishes. Physiological experiments have  
shown that there are substances potent to the olfac-  
tory organ emanating from char (Døving et al. 1973,  
Nordengen 1978). Such substances can also in-  
duce differential responses in the cells of the olfac-  
tory bulb of char (Døving et al. 1974).  
In behavioural experiments Høgland & Åstrand  
(1977) have shown that juvenile char preferred wa-  
ter containing scent of individuals of the same  
population. The present experiment we show that  
mature char are attracted to water

scented by smolts of their own population and that a  
source for these odorants is the intestinal contents.  
Evidence is provided that the chars are attracted  
to a chemically isolated fraction of this material  
(Selset 1980).

## MATERIAL AND METHODS

**Experimental fish.** The fishes used for behavioural ex-  
periments were from a genetically clean population of  
anadromous char (*Salmo alpinus* L.) originating from  
Lake Storsjøen near the city of Hammerfest in Northern  
Norway (70°40'N, 23°42'E). This population starts its re-  
turn to freshwater at the end of June, and the migration is  
completed at the beginning of September (Nordeng 1977,  
and pers. comm.). The time for return to the river depends  
on the age of the fish, the veterans returning first and the  
smolts of the year last. The fishes were hatched and raised  
at the Fish Breeding Experimental Station at Sævi, near  
in Western Norway (62°40'N, 13°34'E) where the be-  
havioural experiments were also performed. The 1973 and  
1974 generations were offspring of the wild fish popu-  
lation, while the 1975, 1976 and 1977 generations were all  
second generation in captivity.

Under the conditions at the Fish Breeding Experimental





# behaviour of mature anadromous char (*Salmo alpinus* L.) towards odorants produced by smolts of their own population

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**Key words:** Fish, salmonid, char, behaviour, smolt, attractants, intestinal contents, bile, chemical fractionation, migration pheromone.

homing and migration of salmonids depend on an intact olfactory organ as shown by Finby & Hasler (1954), Groves et al. (1968) (1973). These findings imply that the fishes are led by odour trails back to their spawning rivers. The substances responsible for these trails have been suggested to emanate from the plants and animals characteristic of the home stream water (Selset 1966, Hasler et al. 1978). Nordeng (1971) has proposed that the substances are released by the smolts of the same populations as the returning fishes. Physiological experiments have shown that there are substances potent to the olfactory organ emanating from char (Døving et al. 1973, Selset 1978). Such substances can also induce differential responses in the cells of the olfactory bulb of char (Døving et al. 1974). Behavioural experiments (Heglund & Åström 1971) have shown that juvenile char preferred water containing scent of individuals of the same species. In the present experiments we show that anadromous char are attracted to water

scented by smolts of their own population and that a source for these odorants is the intestinal contents. Evidence is provided that the char are attracted to a chemically isolated fraction of this material (Selset 1980).

## MATERIAL AND METHODS

**Experimental fish.** The fishes used for behavioural experiments were from a genetically clean population of anadromous char (*Salmo alpinus* L.) originating from Lake Skarvavatnet near the city of Hammerfest in Northern Norway (70°40'N, 25°42'E). This population starts its return to freshwater at the end of June, and the migration is completed at the beginning of September (Nordeng 1977 and pers. comm.). The time for return to the river depends on the age of the fish, the veterans returning first and the smolts of the year last. The fishes were hatched and reared at the Fish Breeding Experimental Station at Semrådgren in Western Norway (62°40'N, 8°34'E), where the behavioural experiments were also performed. The 1973 and 1974 generations were offspring of the wild fish population, while the 1975, 1976 and 1977 generations were all second generation in captivity.

Under the conditions at the Fish Breeding Experimental

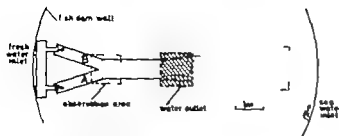


Fig. 1 Sketch showing the test pen for behaviour studies on char used in 1976

Station the char reaches smoltification approximately 1½ year after hatching. After smoltification the chars were allowed to follow their natural annual sea-water/fresh-water cycle with short intermediate periods in brackish water.

Until smoltification the population was kept isolated from other populations. After smoltification the char no longer have the opportunity to sense the smell of fry and smolts of any population.

In addition to artificially raised char smolts from the Hammerfest population anadromous char smolts from Salangen (68°51' N 18°0' E) and stationary char from Trusjoeen Rjukan (59°43' N 9°0' E) were used as odour donor material. The experiments were performed in July/August and at the beginning of September between 17.00 and 01.00 hours. The natural migration takes place between 18.00 and 24.00 hours (Nordeng pers. comm.).

**Experimental pen:** In the behavioural experiments the fishes were given an opportunity to choose between two water currents, one or both of which contained a solution of substances under testing. Several systems were tried two of which are described here. Both systems were situated outdoors in circular concrete fish dams 10 m in diameter painted with dark green Intermacing (International Fact). The water outlets were grids in the center at the bottom of the fish dam.

Equal flow rates of the fresh water current to each pen were achieved by letting water flow into a wooden reservoir of about 220 liters placed just above the upper ends of the canals. The bottom of the reservoir had a round hole (5 cm) for each canal. In order to keep the flow rate constant at 240 l/min in each section the water level in the reservoir was adjusted by means of an overflow system and excess water outlet. The system used in 1976 was a Y-maze made of untreated spruce (Fig. 1). In this system the whole dam was filled with water to a certain level

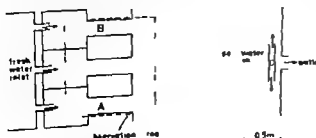


Fig. 2 Sketch showing test pen used in 1977 and 1978

Table 1 Groups of substances tested in behaviour experiments with their concrete canal water

The codes are explained in the text

Test solution	Concentration of dry weight material from the test solutions in canal water (g/l)
Intestinal contents	ca. 10
SKIN mucus	ca. 10 <sup>-2</sup>
Bile	ca. 10 <sup>-4</sup>
HAS-1	1.5 × 10 <sup>-4</sup>
HAS-2	1.7 × 10 <sup>-4</sup>
HAS-3	2.3 × 10 <sup>-4</sup>
HAS-2-A 1	1.5 × 10 <sup>-4</sup>
HAS-2 A	1.5 × 10 <sup>-4</sup>
HAS-2 A 3	1.5 × 10 <sup>-4</sup>
HAS 2 A-4	1.5 × 10 <sup>-4</sup>
(Combined tests)	
HAS-2-B-1	1.5 × 10 <sup>-4</sup>
HAS 2-B 1	1.5 × 10 <sup>-4</sup>
HAS-2-B 3	1.5 × 10 <sup>-4</sup>
HAS-2-B 4	1.5 × 10 <sup>-4</sup>
HAS-2-B 5	1.5 × 10 <sup>-4</sup>
HAS-2-B-6	1.5 × 10 <sup>-4</sup>

while with the second system only the test pen over water. The second pen (Fig. 2) used in 1977 and 1978, made of laminated wood but with canals of grey PVC wooden surface was painted with green Intermacing (International Fact) and the bottom of the canals were white to facilitate detection of the fishes. The second system had three fresh water canals. The one in middle was used to secure good separation of the fish trails from the other two canals. This was confirmed colour tests. The Y-maze did not give as good a section. Most of the test compartment in the second pen was covered with dark plastic to provide protection against disturbance in the choice situation. At the upper end of canals small wooden boxes (pools) were placed to make easier for the fishes to turn. The water level was kept 15–20 cm in the test compartment of both systems means of overflow arrangements.

**Water source:** The freshwater used in the pens and canals came from a nearby river (Lindeløkk) through PVC pipelines. The mature char were kept water from this source during the winter. The water in lower downstream part of the pens was kept brackish means of a sea water inlet (Figs. 1 and 2) with a flow of about 250 l/min in attempts to simulate natural conditions.

**Test solution:** The solutions to be tested were put via polyethylene tubing to the water-streams from 5 l polyethylene containers positioned above the pens. By means of magnetic valves the flow of test solution was controlled from a room situated outside the dam. Test solution were added to the water currents at a rate about 35 ml/min. The turbulence brought about these mixing which was visualized colour.



Fig. 3 The variation in activity of char in the test pen during the period from 17 00 to 01 00 hours in June, July and August in 1976. Each entry gives the mean number of trials per test period of 15 min. The number of test periods are indicated to the right of the entries.

horizontal fractions tested and their concentrations in smolt are listed in Table 1. Besides the chemical and physical contents, skin mucus and bile were also possible attractants to live fish of different strains as tests were also performed with a solution of food in used in smolt feeding. The solution was prepared

30 ml of dry pellets partly dissolved in 3 liters of water and filtered. This solution is approximately 10 000 times diluted in the water currents.

Tests are performed with living smolts as odour sources. This was done by keeping from 3 to 6 smolts in a tank placed just outside the test pen, and an empty tank placed just inside the test pen, and an empty tank placed just outside the test pen, and an empty tank placed just inside the test pen. The concentration of solutions given off by the smolts is unknown. The concentrations of the solutions containing skin mucus and mucus contents were estimated indirectly and are uncertain. The concentration of the bile solution was estimated from fish bile values and is likewise uncertain.

Activity of smolts. The fish were observed by means of an infrared television system (Grundig Electric IP 40) covering the observation area divided in 3 and 2. Whenever complete darkness prevailed, two infrared lamps, each of 300 W mounted symmetrically at 1.5 m above the water surface were turned on. Since the test pen is situated outdoors, the illumination level of the smolts is high. It should be noted that the visual light conditions at Hammerfest are quite different from those at Sandnessjøen.

The fish are considered to have made choice of preference, when the most of the fish reached the marked by tape, stretched over the top of the canal (Figs 1 and 2). Automatic registration with infrared cameras of the fish entering the canals was attempted,

but the results obtained by using these devices were found to be unreliable in the present experiments.

Experimental procedure. The solution to be tested was introduced first into canal A (Figs 1 and 2) for 15 min, usually there was no solution added to canal B. The flow of solution was then stopped for 5 min. In the next 15 min the solution was added to canal B followed by a new pause of 5 min. This 40 min sequence was repeated until a sufficient amount of data had been obtained or no more of the test solution was available. The whole procedure constituted a test session for that particular solution. The number of fishes kept at the same time in the pen varied from 19 to 24 for the first system (1976) and from 5-10 for the second system (1977-1978). Experiments with only one fish in the pen were tried, but found inappropriate because of low efficiency. The fishes could move freely up and down the canals, one or more at a time.

Data treatment. In the experiments it was considered a trial whenever a fish entered one of the canals as described above. Since only two canals were considered for entrance and since the probability for the fish to choose one of the two canals was expected to be 0.5 and constant the binomial model was chosen for the probability calculation (see results). The results are presented as the 'response value'. The term expresses the number of fishes which have chosen the canal containing the test substance, i.e. successful trials divided by the total number of trials. The response value can theoretically vary between 0 and 1, which will signify strong attraction and repulsion, respectively. The final response value obtained in a test session considering all trials would determine the probability of attraction. These values are presented in Table 2. An extract of the table is presented in the preceding article (Sebert 1980) to facilitate the understanding of the course of chemical fractionation. We

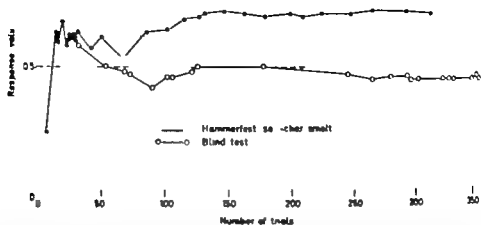


Fig. 4 The response value for mature char towards the odour of their own smolts (●) and towards neutral water added as a test solution (○). In this and the following figures the horizontal stippled line in the diagrams represents a response value of 0.5 indicating no preference. The dotted line represents approximately the response values with the probability of 1%. Some of the entries represent more than one test period due to overlap in the drawings.

consider such tabulated results to hide information about the development of the behaviour tests that should not be concealed from the reader. In the result section graphic representation of the response values are presented in diagrams showing the cumulative results after every 15-min test period. In this way results showing attraction will not be masked by later results from periods when the fishes may not be interested. Due to the drawing technique overlapping entries are represented by one symbol. In the diagrams the curve representing a probability limit of 1% is indicated. Normally a test solution was considered attractive if the response value curve exceeded the 1% level.

**Environmental factors.** In order to enable prediction of the active periods of the fishes, several environmental parameters possibly connected with their activity were routinely noted at the start of every test. These parameters were water and air temperature, such weather conditions as rain, cloudiness, wind and light intensity. Influence on activity by tide and gravity force changes induced by the tide water acceleration force was possible but could not be satisfactorily examined because the tests were performed almost exclusively between 17:00 and 01:00 hours.

## RESULTS

The fishes were accustomed to the artificial breeding environment and seemed to readily adjust to the test pens. During the experimental period the fishes showed wide variation in activity and willingness to enter the canals and thus to produce a

trial. Activity in this respect is presented in Fig. 1, the mean number of trials per test period of 15 min and shows a maximum at about 19:00 hours. The mean number of trials varied greatly from day to day.

**Responses to live fish.** The response of the char to the odour of live smolts was tested. An example of the results from the test session made in 1978 is shown in Fig. 4. In this session Hammerfest char of three years of age was tested as regards response to the odour of their own smolts. The response was indifferent at first trials but after the initial periods the results show a clear attraction. The results of similar tests in 1978 were not as convincing as those from 1977 but still statistically significant at 1% level. Indifferent response to the odour of live smolts was never observed.

**Controls.** Several experiments were performed to observe the behaviour of the fishes with no added test solution. In Fig. 4 is shown the reaction of the two test canals when neutral water was added as a test solution. In this blank session 10 periods of 15 min were performed. During this session the fishes made 357 trials of which 162 were made to the canal "containing" the blank. Of 357 fishes (trials) 177 and 180 entered the A and B canals respectively. The results are consistent with the assumption that the probability for the fish to enter one of the two canals is 0.5.

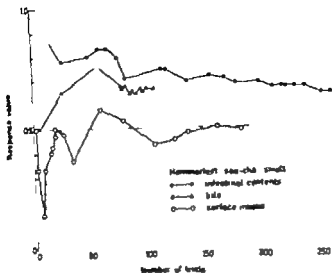


Fig. 5 The response value for mature char towards smolt material: intestinal contents (●), bile (▲) and surface mucus (○).

could be argued that any attractive effect of the mucus or secretion from the fishes might be due to attraction caused by food remains in the gut.

It is hardly likely since fishes like any other animal must be expected to know the difference between food and definite non-food. In any case, pellets of food pellets normally given to smolts were tested. The results show that, at one stage, the response value actually passed the 1% level, but in long run ended up at 0.5. The fishes used for this experiment were 2½ years of age and accepted food pellets given to them. However, in most experiments older fishes were used, and they showed no response to food pellets. This was studied and controlled every year.

**Response to oral secretions from smolt.** Experiments with smolts as odour donors showed that mature sea char were attracted to substances excreted by the smolts. In different seasons of experiments the mature sea char were studied with respect to their reactions to secretions produced by smolts. The materials which were studied were intestinal contents, skin mucus and bile from the gall bladder. The results of one session of tests with these materials are compiled in Fig. 5. Skin mucus, collected with the vacuum cleaning method (Fabell & Setset 1980) did not attract the mature char. The results from one session in 1977 can be seen in the Fig. 5 diagram where the response value in 34 test periods giving 182 trials is summarized. In

the 1976 experiments, the mucus seemed to be attractive but this mucus was collected by a method known to give heavy contamination with intestinal contents (Stabell & Setset 1980). The same year another mixture of mucus and intestinal contents gave no attraction. This negative result remains unexplained.

The intestinal contents collected by the method described by Setset (1980) elicited strong attraction responses in mature char. The results from the test session with the same mature fishes as used for tests with skin mucus are shown in the Fig. 5 diagram. In one test session, the response to intestinal contents was indifferent, but the material had then been collected from starved smolts which were later confirmed as sick.

Since the intestinal contents attracted the fishes and since the attractants were suspected to be some kind of bile acid derivative (Setset 1980) a series of experiments were performed in 1978 to see if bile from the gall bladder of the smolts was attractive to the mature char. The results of these tests are shown in Fig. 5. In test period numbers 5 and 6 represented by the 2nd and 3rd triangle in the figure, the bile seemed to have a powerful attraction. The results of these two tests brought the response value beyond the 1% level. In the following tests the fish behaved indifferent to the bile. However the response value stayed above the 1% level due to the cumulative presentation of the results.

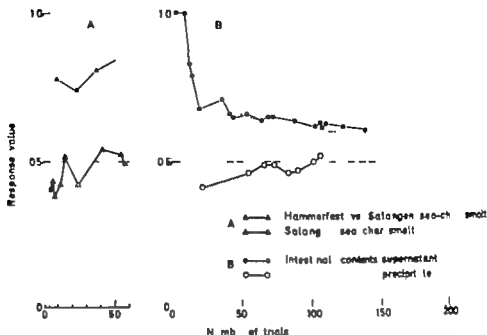


Fig. 6 The response values for mature Hammerfest char (A) Towards Hammerfest smolt intestinal contents versus Salangen smolt intestinal contents (A) and towards Salangen smolt intestinal contents alone (△) (B) Towards supernatant (HAS) and precipitate (HAB) of intestinal contents from Hammerfest char smolts (● and ○ respectively)

#### Responses to different char populations

Whether or not the attractive substances were specific to the Hammerfest population of char was planned to be investigated thoroughly but this was prevented by new quarantine regulations at the Fish Breeding Experimental Station. However competition tests were performed with intestinal contents from smolts of a different population of anadromous char from the Salangen river system. Test solutions of intestinal contents from Hammerfest smolts and Salangen smolts were prepared from one fish each. A preference for the sample from its own population was observed as can be seen from the diagram in Fig. 6A. This result may be suspected as partly due to a repellent effect from the strange smell. Therefore the sample of Salangen material was tested alone but showed no repulsive effect (Fig. 6A). However the intestinal contents of the Salangen smolts were transported by mail to Sundalsøra and possible attractants could have been destroyed by microbial processes in the time lag.

Another competition test was performed using live Hammerfest smolts and 2½ year old stationary chars (Tinnsjø Rjukan) as odour donors. These tests were conducted in 1977 right after the very convincing experiments with smolts alone. The results showed an almost significant (1% level) attrac-

tion of the mature Hammerfest chars to the Tinnsjø freshwater population. This puzzling result could be explained in two ways. The attraction could be caused by some kind of conditioning since Tinnsjø chars had been kept together with the 1973 and 1974 generations of Hammerfest chars for 1½ years before the experiment. The attraction could also possibly be explained by sex attraction since the Tinnsjø chars seemed to be old (beautiful spawning colours) in spite of low age. Since the smolts alone were so attractive to mature chars just before the strange population was judged to be at least as attractive as the smolts.

Among the fractions of the intestinal contents described in the preceding article (Sæset 1976) a convincing attraction effect was induced by supernatant (HAS) whereas the precipitate (HAB) gave no response (Fig. 6B). These tests were carried out in 1976 with a starting material that was a mixture of intestinal contents and surface material. Of the gel filtration fractions of this material the one corresponding to HAS-2 was judged to be attractive. This fraction is among the retarded ones which have been shown to originate almost exclusively from intestinal contents (Ståbø & Sæset 1980). Since then only intestinal contents have been fractionated further.

bouring fraction might also be considered weakly attractive. All the HAS-2-B fractions were tested at a concentration of  $1.5 \times 10^{-8}$  g/l.

The fractions of HAS-2 A (one through four) were pooled to be tested as one solution to save time. These fractions seemed not to elicit significant attraction in the behavioural studies as can be seen from the diagrams in Fig. 8. However it should be kept in mind that the number of trials made in some of these behavioural tests was low due to paucity of available test material.

## DISCUSSION

The result of the present experiments has shown that the macromous char of a population from Northern Norway is attracted to the scent of smolts from its own strain. The experiments have also provided evidence that the substances are released with the intestinal contents and might possibly be found in the bile of the gall bladder. Each of these points raises a number of questions and will be discussed. The experimental test situation also requires consideration, and finally the implications of the present results in the light of the pheromone hypothesis proposed by Nordling (1971, 1977) will be discussed.

The behavioural experiments meet with all the requirements for the binomial model, except for one. The probability for the fishes to choose a cer-

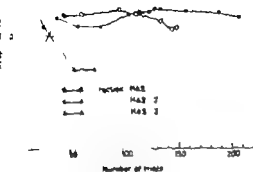


Fig. 7. The response values for mature char towards fractions of the supernatant (HAS) of intestinal contents, HAS-1 ( $\Delta$ ), HAS-2 ( $\circ$ ) and HAS-3 ( $\square$ ).

The presence of attractive substances in the HAS-fraction were confirmed by the experiments of 1977 when HAS-1, HAS-2 and subfractions of HAS-2 were tested. The results of these experiments are presented in Fig. 7 and 8. HAS-1 could possibly turn out to be attractive but corresponding fractions did not give attraction in the 1976 experiments.

The only subfraction of the HAS<sub>2</sub> judged to be attractive was HAS-2-B-3. HAS-2-B-4, the neigh-

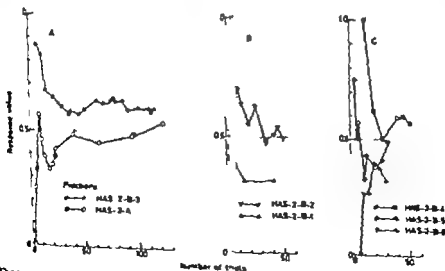


Fig. 8. The response values of mature char towards the fraction HAS-2 A and the subfractions of HAS-2-B. See text.



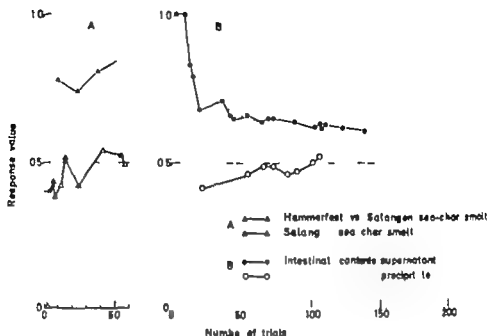


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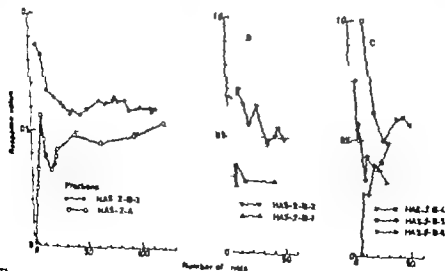


Fig. 8. The response values of mature char towards the fraction HAS-2-A and the subfractions of HAS-2-B. See text.

Table 2. Final data for the behaviour tests

The probability values are correct only if the binomial model is valid for the experiments

Test solution	Year tested	No of test periods	Total no of trials (N)	No of successful trials (Y)	Probability in % for 1 or more successful trials of N trials
Hammerfest sea-char smolt	1977	38	313	219	<0.01
Hammerfest sea-char smolt	1978	38	153	97	0.76
Hammerfest sea-char smolt					
Intestinal contents	1976	23	978	156	2.4
Intestinal contents	1977	4	261	185	<0.01
Intestinal contents	1978	30	155	98	0.07
Hammerfest sea-char smolt surface mucus	1977	34	183	90	56
Intestinal contents supernatant (HAS)	1976	22	218	123	3.4
Intestinal contents precipitate (HAB)	1976	8	105	55	33
HAS-1	1977	8	150	79	38
HAS-2	1976	12	210	119	3.1
HAS-3	1977	4	71	25	99*
HAS-2 A	1978	18	123	66	4
HAS-2-B 1	1978	10	42	13	99*
HAS-2-B 2	1978	14	41	25	50
HAS-2-B 3	1978	24	114	68	4
HAS-2-B 4	1978	8	51	79	20
HAS-2-B 5	1978	8	33	16	40
HAS-2-B 6	1978	8	31	10	96
Hammerfest sea-char smolt, bile	1978	28	108	71	0.08
Food pellets	1977	26	294	148	48
Hammerfest vs. Salangen sea-char smolt					
Intestinal contents	1978	4	51	45	<0.01
Salangen sea-char smolt Intestinal contents	1978	10	57	28	50
Hammerfest smolt vs. Tinnøya char	1977	30	194	80	99*
Blank test	1976	26	357	162	96

\* Indicates repellent effect of the test solution

\* Indicates that the odour from the strange population is preferred

tain canal seemed to be variable. Periodic preference for one of the canals was observed. This preference could be for either canal and last up to several hours. This could however be caused by internal social relations between the fishes in the test compartment. For instance, if a hierarchy is established, the position of the leader fish within the system could easily have consequences for canal selection by the other fishes. Therefore, the ideal way of testing would be to use only one fish at a time. As mentioned earlier, however, this was not practical. To perform the experiments with the systems used, to reduce the necessary number of trials for each test solution, the number of canals arranged in a symmetrical way should be extended. In some behaviour experiments, it would be suf-

ficient to let the fishes perform a certain number of trials and then calculate the probability and chance of the final result. However, to conduct experiments in this way, one needs additional information on fish behaviour. For example, what the fishes will be searching for: the possible "pheromones" constantly or only at certain periods of the day. In the latter case, one would need assurance that the tests were performed only at the right periods. Another question is whether or not the migrating instinct will be "switched off" once the fishes have reached fresh water containing the pheromone. The development of the trials of a session is therefore given in graphs.

The results of the experiments with the fraction of intestinal contents are not as evident as the

the crude intestinal material itself. However total results add up to a fairly reliable anticipation of the attractive substance(s) to be found in the fraction HAS-2-B-3. This judgment was strengthened by the fact that in physiological experiments the olfactory organ had a threshold (50 or lower to this fraction than to any of the others (Mag et al. 1979 unpublished). The threshold measurements were performed after the last series of behavioural experiments. A weak behavioural response to a pure attractant could be suspected if fish that are exposed to a single odorant without the natural odorous background could become provoked and confused. The local river from which the water was taken contains no charr. A complete isolation of the natural response could perhaps be reached. A weak response to the pure attractant could also be expected if several substances were involved in a synergistic way to induce full response in natural conditions.

The experiments with live smolts showed that the mature charrs are attracted to smolts from their own population at the time for natural homes and migration.

The competition experiments suggest that the active substances might be specific for the immature population of charr. The results from competition tests with Hämmerfjell smolts and more Tinnsjø charr could be evidence of an interesting phenomenon, but the explanation that the fish were attracted to some sexual component is probable.

The behavioural experiments evidence that all active substances are found in the intestinal contents. The skin mucus has been shown to contain most olfactory substances (Düring et al. 1973, 74) but this material might have been contaminated with intestinal contents either directly by the sampling procedure or indirectly since these substances will be present in the water and might be absorbed by the skin mucus. The skin mucus has often suggested to be the source of the "migration" hormones (Nordeng 1971), but the present experiments suggest other sources. However "pure" skin mucus was only tested once and cannot therefore be totally excluded as source of attractants. In addition the migration hormones may not function in the artificial situation of the behavioural experiments.

If the attractants are found in the bile, as the present indicates, the chemical isolation of the substances would be greatly simplified. This would

also mean that these substances are likely to be synthesized in the liver. However there is still the possibility that the attractants are produced by intestinal micro-organisms and returned to the bile via the entero-hepatic circulation.

The results of the present study support the pheromone hypothesis presented by Nordeng (1971, 1977) which explains the migration of salmonids from their hatching areas in the river system out to sea and back to their home spawning grounds. His observations and experimental findings can most reasonably be explained by the presence of substances from the smolts of the same population released into the water in form of the trail by which first the mature fish, and then the smolts, can follow back to the spawning grounds.

Our results however do not exclude an olfactory imprinting, but make it likely that the possible "imprinting" would be to substances emanating from the fishes themselves and not from vegetation or minerals (Hasler 1966). An optimal way to elucidate the mechanism of salmonid migration could be to identify the guiding substances and their natural source and thus to enable imprinting experiments with the natural group of substances.

This study was supported by the Norwegian Fisheries Research Council. We are indebted to T. Gjerdén, A. Kavelaas, T. Refsum, K. Gundersen and the staff of the Fish Breeding Experimental Station at Sandnessjøen for their cooperation and helpful advice. We also wish to thank O. B. Sæviell, B. Flåtten and G. Aasen for their assistance.

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# factory sensitivity to bile acids in monid fishes

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Monopolar DC-recordings were made simultaneously from two positions on the olfactory bulb of charr (*Salmo alpinus* L.) and graylings (*Thymallus thymallus* L.) using bile acids and amino acids as olfactory stimulants. The bile acids induced responses with characteristic spatial differences from those of the amino acids. The distribution of responses to bile acids indicated a neuronal activity in the medial part of the bulb. In contrast, amino acid elicit responses in the lateral part of the bulb. Taurine conjugated bile acids were up to 1 000 times more potent as olfactory stimuli than methylcysteine. The results suggest that olfactory receptors are of two types, one responding to bile acids the other to amino acids. 3- $\alpha$ -hydroxysteroids are released from the fish into the water in quantities that suffice for detection by their olfactory system. The odorous potency of the bile acids their evolutionary history and variability together with their renowned adherent properties made them interesting candidates for specific signals in the aquatic environment.

**Key words:** Fish, olfactory bulb, induced potentials, spatial distribution, odour potency, bile, bile salts, amino acids, chemical signals, migration

accurate homing of salmonids has been shown to be an olfactory basis (Wisby & Flaker 1954; es et al. 1968; Toft 1975). Odorous substances can provide such an accurate migration over distances and time must have properties that be described in terms of diversity, specificity, and stability. The theory proposed by denig in 1971 for the mechanisms of homing by tonid fishes requires that the odorous substances emanate from fishes in the river system, from smolts on their seaward migration.

Physiological experiments have shown that fishes detect substances that are potent stimuli to the olfactory organ (Døving et al. 1973) and that there is a neuronal basis for discrimination of different strains of fishes (Døving et al. 1974). By recording from the bulbar surface of salmonids, Thommesen (1973) showed that amino acids used as olfactory responses in the lateral part of the bulb in water containing fish elicited responses in the lateral and the medial part of the bulb. At that time chemical nature of the substances that elicited responses in the medial parts were unknown.

In the present study we show that bile acids evoke responses in the medial part of the bulb. The majority of pure bile acids tested, elicited distinct responses in concentrations below the ones usually considered to be thresholds for amino acids. The possible function as odorants of this group of chemical compounds is discussed. The results of the present study have been presented previously as a short communication (Døving, Selset & Thommesen 1978).

## MATERIAL AND METHODS

The experimental fishes used in the present study were anadromous charr *Salmo alpinus* (L.) syn. *Salvelinus alpinus* (L.) and grayling *Thymallus thymallus* (L.). The charr were raised at the Fish Breeding Experimental Station, Senndalsøra, Western Norway but originated from the Hammerfest region of Northern Norway. The graylings were caught in small creeks of Lake Mjøsa in Southern Norway. The weights of the fishes were between 200 and 500 g. The fishes were transferred to the aquarium facilities at the University of Oslo before use. In the experiments the fishes were immobilized with 4-tubocurarine

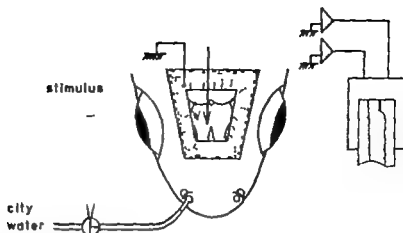


Fig. 1 Schematic diagram of the stimulation and recording arrangements. The relative size of olfactory bulbs is exaggerated

(Nyegaard & Co. Oslo) 10 mg/kg i.m. The fish was wrapped in a wet sponge and maintained in a fish holder under continuous flushing of the gills with tap water at 5–8°C. The dorsal part of the brain case was opened and the olfactory bulbs exposed (Fig. 1). During the surgical procedure the fish was usually anaesthetized with MS 222 (Sandoz) by infusion into the respiratory water flow to a final concentration of 100 ppm (mg/l). Exceptions were fishes serving as controls.

**Recordings.** The potentials were picked up by use of glass capillaries filled with a physiological salt solution. The electrodes were inserted into electrode holders containing slanted Ag/AgCl. Electrode tip diameter ranged from 20–100  $\mu$ m. The electrode tips were placed on the dorsal surface of the olfactory bulb. The potential changes were recorded using preamplifiers with frequency response DC–70 Hz and monitored on a pen recorder. All recordings were monopolar and the preparation was grounded through the indifferent electrode only which was placed on the cranium just posterior to the opening in the skull (Fig. 1).

**Stimulation.** The stimuli were introduced from a polyethylene pipette through a continuous flow of aquarium water into the ipsilateral anterior nares and controlled by a 3-way stopcock. Nasal water flow was kept at 0.3 ml/s.

Fish basin water samples, bile from both species and the 11 substances listed in Table 1 were used as stimuli.

The chemical composition of bile acid determinants by the drawing of cholanic acid in Fig. 2 and the  $pH$  of the added groups listed in Table 1. All stimuli diluted in decadic steps, the pure substances from  $10^{-1}$  to  $10^{-9}$  M, the bile in dilutions from  $4 \times 10^{-4}$  to 1 vol/vol.

**Experimental procedure.** Responses have been recorded from both olfactory bulbs stimulating the ipsilateral olfactory epithelium. The recording electrodes placed in different positions on the dorsal surface of bulb. Responses from a medial and a lateral position recorded simultaneously in each experiment.

The blood flow in the brain surface vessels was carefully inspected as a control of circulatory condition.

Each kind of stimulus was presented in the ascending concentration, each concentration tested twice consecutively. The order of the stimuli varied at random. To avoid fatigue stimulation intervals were at least 30 s or as long as any sign of the response remained. Stimulation time was marked orally and later adjusted for the time lag present due to construction of the stimulation device. Each position was initially checked by testing the response to methionine, sulfolauric, thiocholic acid and a water sample from the fish basin.

**Evaluation of responses.** In principle evaluation of magnitude of the bulb surface response involves

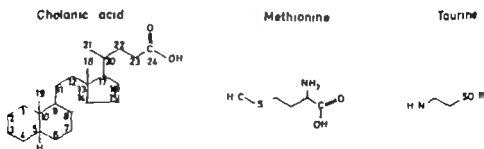


Fig. 2 Chemical structure of the stimulating substances. For the position of substituents on the cholanic acid skeleton see Table 1.

1. Threshold values of pure compounds  
compare with Fig. 2

on page	Position of groups			Mean threshold value (M)	Threshold value range (M)
	OH	SO <sub>3</sub> H	Tau		
acetic acid	3 7 14			$1.0 \times 10^{-4}$	$10^{-4}$ – $10^{-5}$
choleic acid	3 7 12		24	$2.0 \times 10^{-5}$	$10^{-4}$ – $10^{-5}$
oleic acid	3 12				—
deoxy-choleic acid	3 12		24	$6.3 \times 10^{-5}$	$10^{-4}$ – $10^{-5}$
choleic acid	3 7				—
choleic acid	3 7		24	$4.8 \times 10^{-5}$	$10^{-4}$ – $10^{-5}$
choleic acid	3				
methionine	3		24	$6.3 \times 10^{-5}$	$10^{-4}$ – $10^{-5}$
choleic acid		3	4	$1.0 \times 10^{-3}$ $1.3 \times 10^{-4}$ $1.0 \times 10^{-3}$	$10^{-4}$ – $10^{-5}$ $10^{-5}$ – $10^{-4}$

replaced not determined due to low solubility

various of DC shifts and AC amplitudes independent. However, for each type of stimulus the size of these responses is proportional to the concentration of the stimulus (Thommesen 1978). In several works it has therefore concentrated on the deflection as being the more rapidly measured parameter.

DC potential shifts were measured in the nose of the fish as 3–4 after the onset of the stimulus. Due to the general instability of the DC recording the measurements of the DC deflection are inaccurate.

90  $\mu$ V

## RESULTS

As reported in previous investigations, the electrical activity recorded from the surface of the olfactory bulb in fishes exhibits irregular and spontaneous fluctuations (Dovling et al. 1973; Dovling & Bell 1977; Thommesen 1978) comparable to those of the frog (Otteson 1976b). Stimulation with a constant odorant induced changes in the pattern of the electrical activity. These responses of the olfactory bulb consist of a slow rise in potential upon which rhythmic oscillations of induced waves are superimposed (Adrian 1940; von 1976a, b). Some examples of these recordings are seen in Fig. 3. To prepare these recordings the dorsal surface of the olfactory bulb was

searched to find the areas most sensitive to sulfotaurine/choleic acid and methionine respectively. The recordings shown in Fig. 3 are taken from a series of experiments where the responses at these positions of the bulb were investigated with different concentrations. As seen, there is a slight change in DC potential of the recording trace when stimulating with sulfotaurine/choleic acid at a concentration of  $10^{-12}$  M; clear induced waves are not seen. When stimulating with a concentration 10 times higher there was a distinct DC potential shift together with clear indications of induced waves. Whenever found the induced waves appeared at the same concentration step where a significant DC potential shift was seen.

**Concentrations.** The results shown in Fig. 3 are typical of the responses obtained in areas of the olfactory bulb reacting to each of the two substances. The amplitude of the DC shift increased with the concentrations as did the amplitude of the induced waves. The maximum amplitudes of the DC shifts were about 1 mV; the induced waves could be in the order of 0.4 mV peak to peak at most. The variations of the AC and DC responses in relation to the concentrations have been described by Thommesen (1978). These results indicated that the neuronal pool activated by the odorous stimuli



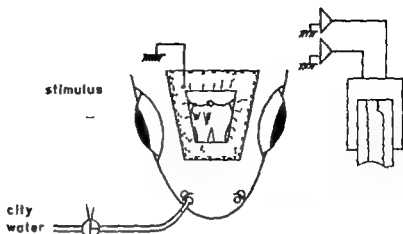


Fig. 1 Schematic diagram of the stimulation and recording arrangements. The relative size of olfactory bulbs is exaggerated.

(Nyegaard & Co. Oslo) 10 mg/kg i.m. The fish was wrapped in a wet sponge and maintained in a fish holder under continuous flushing of the gills with tap water at 5–8°C. The dorsal part of the brain case was opened and the olfactory bulbs exposed (Fig. 1). During the surgical procedure the fish was usually anesthetized with MS 222 (Sandoz) by infusion into the respiratory water flow to a final concentration of 100 ppm/(mg/l). Exceptions were fishes serving as controls.

**Recordings.** The potentials were picked up by use of glass capillaries filled with a physiological salt solution. The electrodes were inserted into electrode holders containing sintered Ag/AgCl. Electrode tip diameter ranged from 20–100 µm. The electrode tips were placed on the dorsal surface of the olfactory bulb. The potential changes were recorded using preamplifiers with frequency response DC 20 Hz and monitored on a pen recorder. All recordings were monopolar and the preparation was grounded through the indifferent electrode only which was placed on the cranium just posterior to the opening in the skull (Fig. 1).

**Stimulation.** The stimuli were introduced from a polyethylene pipette through a continuous flow of aquarium water into the ipsilateral anterior nares and controlled by a 3-way stopcock. Nasal water flow was kept at 0.3 ml/s.

Fish basin water samples, bile from both species and the 11 substances listed in Table 1 were used as stimuli.

The chemical composition of bile acid depends on the drawing of cholic acid to Fig. 1 and the type of the added groups listed in Table 1. All compounds were diluted in decadic steps, the pure substances from 10<sup>-3</sup> M, the bile in dilutions from 4 × 10<sup>-4</sup> M to 10<sup>-5</sup> M.

**Experimental procedure.** Responses have been recorded from both olfactory bulbs stimulating the right olfactory epithelium. The recording electrode placed in different positions on the dorsal surface of the bulb. Responses from a medial and a lateral position recorded simultaneously in each experiment.

The blood flow in the brain surface vessels was carefully inspected as a control of circulatory conditions.

Each kind of stimulus was presented in the ascending concentration: each concentration was tested twice consecutively. The order of the stimuli varied at random. To avoid fatigue stimulation time were at least 30 s or as long as any signs of the response remained. Stimulation time was marked on a pen recorder and later adjusted for the time lag present due to construction of the stimulation device. Each position was initially checked by testing the response to methionine, sulfotaurine, cholic acid and a water sample from the fish basin.

**Evaluation of responses.** In principle evaluation of the magnitude of the bulb surface response involves

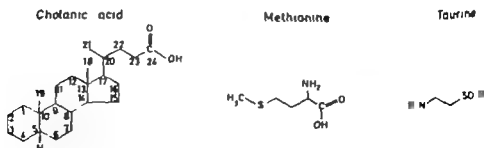


Fig. 2 Chemical structure of the stimulating substances. For the position of substituents on the cholic acid skeleton see Table 1.

1. The *threshold values of pure compounds*  
structure, compare with Fig. 2

ion name	Position of groups			Mean thresh- old value (M)	Threshold value range (M)
	OH	SO <sub>3</sub> H	Trim		
acid	3 7 12	-	-	8.0 $10^{-6}$	$10^{-6}$ $10^{-5}$
choleic	3 7 12	-	24	2.0 $10^{-6}$	$10^{-6}$ $10^{-5}$
ycholic	3 12	-	-	-	-
choleic or acid	3 12	-	24	6.3 $10^{-6}$	$10^{-6}$ $10^{-5}$
choleic or acid	3 7	-	-	-	-
choleic or acid	3 7	-	24	4.0 $10^{-6}$	$10^{-6}$ $10^{-5}$
choleic or acid	3	-	-	-	-
choleic or acid	3	-	4	6.3 $10^{-6}$	$10^{-6}$ $10^{-5}$
choleic or acid	-	3	24	1.0 $10^{-6}$ 1.5 $10^{-6}$ 1.0 $10^{-6}$	$10^{-6}$ $10^{-5}$ $10^{-6}$ $10^{-5}$

threshold not determined due to low solubility

measurements of DC shifts and AC amplitudes independent. However, for each type of stimulus the size of these two components are proportional as long as the ring positions are maintained (Thommesen 1978). In present work we have therefore concentrated on the deflection as being the more rapidly measured parameter. The DC potential shifts were measured as the mean action of the tracing at 3-4 s after the onset of the case. Due to the general instability of the DC recording the measurements of the DC deflections are inaccurate to  $\pm 50 \mu V$ .

## RESULTS

reported in previous investigations the electrical activity recorded from the surface of the olfactory bulb of fishes exhibits irregular and spontaneous fluctuations (Davies et al. 1973; Davies & Belgig 1977; Thommesen 1978) comparable to those found in the frog (Otteson 1959b). Stimulation with or containing an odorant induced changes in the variance of the electrical activity. These responses of the olfactory bulb consists of a slow surface potential upon which rhythmic oscillations induced waves are superimposed (Adrian 1950; Otteson 1959a, b). Some examples of these recordings are seen in Fig. 3. To prepare these recordings, the dorsal surface of the olfactory bulb was

sought to find the areas most sensitive to sulfotaururothiocholeic acid and methionine respectively. The recordings shown in Fig. 3 are taken from a series of experiments where the responses at these positions of the bulb were investigated with different concentrations. As seen there is a slight change in DC potential of the recording trace when stimulating with sulfotaururothiocholeic acid at a concentration of  $10^{-6}$  M; clear induced waves are not seen. When stimulating with a concentration 10 times higher there was a distinct DC potential shift together with clear indications of induced waves. Whenever found, the induced waves appeared at the same concentration step where a significant DC potential shift was seen.

**Concentrations.** The results shown in Fig. 3 are typical of the responses obtained in areas of the olfactory bulb reacting to each of the two substances. The amplitude of the DC shift increased with the concentrations, as did the amplitude of the induced waves. The maximum amplitudes of the DC shifts were about 1 mV; the induced waves could be in the order of 0.4 mV peak to peak at most. The variations of the AC and DC responses in relation to the concentrations have been described by Thommesen (1978). These results indicated that the neuronal pool activated by the odorous stimuli

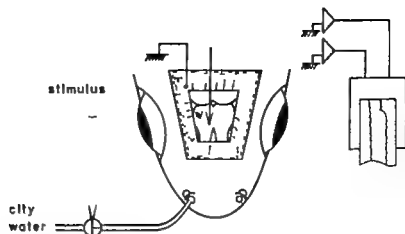


Fig. 1 Schematic diagram of the stimulation and recording arrangements. The relative size of olfactory bulbs is exaggerated.

(Nyegaard & Co. Oslo) 10 mg/kg i.m. The fish was wrapped in a wet sponge and maintained in a fish holder under continuous flushing of the gills with tap water at 5–8°C. The dorsal part of the brain case was opened and the olfactory bulbs exposed (Fig. 1). During the surgical procedure the fish was usually anesthetized with MS 222 (Sandoz) by infusion into the respiratory water flow to a final concentration of 100 ppm/(mg/l). Exceptions were fishes serving as controls.

**Recordings.** The potentials were picked up by use of glass capillaries filled with a physiological salt solution. The electrodes were inserted into electrode holders containing sintered Ag/AgCl. Electrode tip diameter ranged from 20–100 µm. The electrode tips were placed on the dorsal surface of the olfactory bulb. The potential changes were recorded using preamplifiers with frequency response DC–100 Hz and monitored on a pen recorder. All recordings were monopolar and the preparation was grounded through the indifferent electrode only which was placed on the cranium just posterior to the opening in the skull (Fig. 1).

**Stimulation.** The stimuli were introduced from a polyethylene pipette through a continuous flow of aquarium water into the ipsilateral anterior nares and controlled by a 3-way stopcock. Nasal water flow was kept at 0.3 ml/s.

Fish basin water samples, bile from both species and the 11 substances listed in Table 1 were used as stimuli.

The chemical composition of bile acid derivatives is given by the drawing of cholic acid in Fig. 2 and the position of the added groups listed in Table 1. All stimuli were diluted in decadic steps; the pure substances from  $10^{-10}$  to  $10^{-18}$  M, the bile in dilutions from  $4 \times 10^{-10}$  to 1 vol/vol.

**Experimental procedure.** Responses have been recorded from both olfactory bulbs stimulating the ipsilateral olfactory epithelium. The recording electrodes were placed in different positions on the dorsal surface of the bulb. Responses from a medial and a lateral position were recorded simultaneously in each experiment.

The blood flow in the brain surface vessels was carefully inspected as a control of circulatory conditions.

Each kind of stimulus was presented in the ascending concentration; each concentration was tested twice consecutively. The order of the stimuli varied at random. To avoid fatigue stimulation time was at least 30 s or as long as any sign of the pre-response remained. Stimulation time was marked orally and later adjusted for the time lag present due to construction of the stimulation device. Each preparation was initially checked by testing the response to methionine, sulfatauroallocholic acid and a water sample from the fish basin.

**Evaluation of responses.** In principle evaluated magnitude of the bulb surface response in the

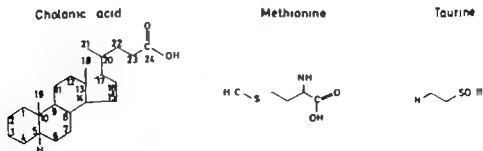
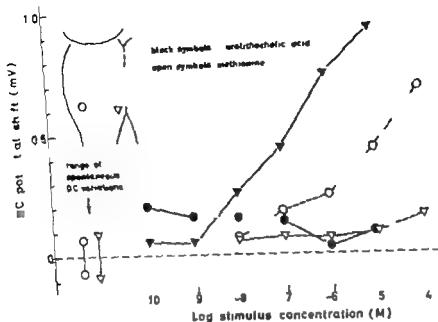


Fig. 2 Chemical structure of the stimulating substances. For the position of substituents on the cholic acid skeleton see Table 1.



1. Diagram of the relation between the behavior responses and stimulus strength. The magnitude of DC potential shifts responses to methionine and taurithiocholic acid are plotted against the different concentrations.

ness occurred between methionine and taurithiocholic acid.

**hemical structure and response threshold** The free unconjugated bile acids were not as potent odorants as the conjugated ones. The mean sensitivity for the different compounds are given in Table 1. For comparison the table includes the sensitivities for methionine and taurine as well. The free conjugates and especially the sulfonated ones regularly a lower threshold for eliciting responses in the olfactory system than any of the conjugated derivatives. The absolute sensitivity varied for the different preparations, but the rank followed nearly the same order in all experiments. Some of the bile acids derivatives have so low solubilities that their thresholds could not be satisfactorily determined. These were therefore excluded from further investigations. These substances are marked with an asterisk in Table 1.

**Crude products** Crude bile taken from the abdominal of the two species used in the present experiments was tested. Responses were obtained at dilutions down to about  $1:10^6$ . Distribution of responses to the bile was similar to that of the pure acids.

Seawater containing fish gave responses both

in the medial part of the olfactory bulb i.e. at the same area as the bile acids, and in the lateral part (Thommesen 1978). The presence of bile acids in basin water was investigated by holding a char to 10 l of water for 4 h. The water was then analyzed for its contents of 3- $\alpha$ -hydroxysteroids in relation to a control (Sterogonul 3- $\alpha$  Nyegaard et Co Oslo). The analysis showed an effluence of 3-OH steroids of 34 nmol kg<sup>-1</sup> h. This result indicates that a sufficient quantity of bile acids emanate from the fish to be detectable by the olfactory system of the fish. Artificial mixtures of methionine and bile in different concentrations elicited responses that were qualitatively and quantitatively intermediary between the responses to each of the constituents. It was thus possible to imitate responses to basin water by an artificial mixture of known substances.

Most of the present experiments were performed in May and June 1978 with pilot studies in April and some supplementary tests in August. Late in August, the fishes gradually lost their sensitivity to all olfactory stimuli tried. In January 1979 the fishes regained their sensitivity to basin water samples and to some extent to diluted bile to a lesser extent to the pure substances. The reason for this change in sensitivity is unknown.

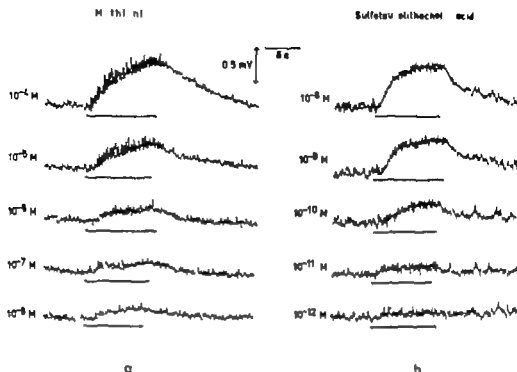


Fig. 3 Responses to different concentrations of methionine and sulfotaurolithocholic acid obtained at their recording positions. Note the different concentration levels in the two series.

increased and/or that the depolarization of the individual neurones and synchronizations of spike activity increased with increasing stimulus concentration.

An example of variation in the amplitude of these responses with concentration of the stimulus can be seen in Fig. 4. The electrode positions are in the anterior region, one lateral and the other medial. As seen the medial part of the bulb responded only to the taurolithocholic acid. The lateral part of the bulb responded to methionine. Responses increased approximately linearly with the logarithm of the stimulus concentration.

**Spatial distribution of responses.** As demonstrated by studies by Thommesen (1978) the responses recorded from the olfactory bulb depend on the electrode positions. This dependence also appears from the results shown in Fig. 4. Two of the recordings from the experimental series making up Fig. 4 are displayed in facsimile in Fig. 5. By recording simultaneously from two regions of the dorsal surface of the olfactory bulb as indicated in the schematic drawing of Fig. 5 the differential sensitivity of the regions is clearly demonstrated. The upper part shows recordings during stimulation with taurolithocholic acid at a concentration of  $10^{-7}$  M while the lower part shows the responses to stimulation with  $10^{-8}$  M of methionine. Taurolitho-

cholic acid gave a large response in the medial of the bulb but hardly any response in the lateral part. In contrast methionine gave a considerable response in the lateral region and hardly any in the medial part of the bulb.

Further information of the spatial distribution of the responses was obtained by exploring the surface with two electrodes and studying the responses to a concentration series of a potent acid and methionine. As response parameters measured the magnitude of the DC potential, confirming the distribution of the response, considering the magnitude and polarity of the induced waves.

**Cross-adaptation.** The spatial isolation of bulbar responses to the two types of stimuli suggests that there are two groups of olfactory receptors responding either to amino acids or to acids. As pilot experiments for receptor cross-adaptation experiments were made in  $10^{-4}$  M of methionine and  $10^{-4}$  M of tauric acid. The bulbar responses were recorded by switching directly from one stimulating substance to the other. Even after 11 min of continuous exposure to one of these stimuli the immediate response to the other was unaltered. Thus apparently no significant cross-adaptation of the bulbar

as with amino acids elicited responses in the lateral part of the olfactory bulb whereas crude fish extract gave responses in both lateral and medial parts.

The results of the present experiments demonstrate that some bile acids elicit responses in medial part of the bulb only.

**Receptors.** The spatial separation of the responses to representatives of the amino acid and the bile acid groups, suggests that the receptors for the two kinds of stimuli should be located in separate receptor cells. Preliminary experiments indicate a lack of cross adaptation between representatives of the two groups of compounds, thus they presumably activate discrete pathways in the olfactory system. One type of receptor cell should therefore be receptors for the bile acids, another for amino acids. In the goldfish there are two morphological types of receptor cells, one with microvilli and another with cilia (Ichikawa & Ueda 1977). The two morphological cell types have been found in Pacific salmon (Yasumoto & Ueda 1977) presumably with the same function. It is an interesting possibility that each morphological type has receptors responding to one of the two kinds of chemical stimuli.

**Receptor distribution.** Since the olfactory bulb is as a relay station between the peripheral olfactory epithelium and the central nervous system, our findings on the spatial distribution of the responses in the olfactory bulb have implications both for the convergence of receptors and the projection of secondary neurones from the bulb. Theoretically the two different kinds of receptor cells, those responding to amino acids and the other responding to bile acids, might either be topographically separated or intermingled in the olfactory epithelium. A topographically separated distribution seems more likely when considering the spatial distribution of the responses at the level of the olfactory bulb. An intermingled distribution might be of advantage with respect to the water sampling of the olfactory organ.

**Central projections.** The secondary neurones of the olfactory bulb project to the central nervous system via distinct bundles that form the olfactory tract (Sheldon 1912; Daving & Gemoe 1965). The projection is arranged so that neurones mainly situated in the lateral part of the bulb constitute the lateral tract and neurones mainly in the medial part form the medial tract (Daving et al. in preparation). This subdivision of the olfactory tract strongly indi-

cates that the bundles serve different functions. The results of the present investigation imply that these functions may be connected to different groups of chemicals.

**Bile acid.** In salmonids the bile acids produced are chiefly the taurine conjugates of cholic and chenodeoxycholic acid (Nagayoshi et al. 1964; Sasaki 1966; Haslewood 1967; Ripatti et al. 1973; Denton et al. 1974). What happens to the bile as it descends the intestine in fishes is largely unknown, but our experiments have shown that anadromous charr release about  $34 \text{ nmol kg}^{-1} \text{ h}^{-1}$  of 3- $\alpha$ -hydroxysteroids into the surrounding water.

The threshold for eliciting bulbary responses to basin water containing fish of the same population is in the range of  $0.03 \text{ fish h}^{-1} \text{ l}^{-1}$  (Thommesen, unpublished results). Each fish weighs about  $0.2 \text{ kg}$ . Thus the threshold is equal to  $0.006 \text{ kg h}^{-1} \text{ l}^{-1}$ .

When considering the production rate of  $34 \text{ nmol h}^{-1} \text{ kg}^{-1}$  the threshold for the fish olfactory system to detect these substances will be about  $2 \times 10^{-10} \text{ M}$  and thus at the same concentration level as the threshold for the more potent of the bile acids. This calculation shows that the production of 3-OH bile acids could account for the bile acid like part of the responses to basin water but does not exclude the existence of other potent odorants.

The olfactory sensitivity of the fishes was shown to vary reaching a minimum during the autumn. A similar loss of sensitivity has been observed in another population of charr during the early part of the winter (Thommesen 1978). One cause for this variation may be a natural change in olfactory sensitivity but a poisoning action of the aquarial water supply could be a possibility. It is most reasonable that this decrease of olfactory sensitivity reflects an annual change that follows the life cycle of these fishes. Whatever the cause may be, this selective anosmia to the pure amino acids and bile acids tested compared with a recovery of the sensitivity to basin water indicates that the basin water may contain potent odorants which are different from the pure substances tested so far. Thus we are dealing with either a third class of odorant receptors in the epithelium and/or annual changes in olfactory sensitivity to specific substances.

The evolutionary history of the bile acids in vertebrates has been reviewed by Haslewood (1967). He summarized the vast amount of findings of the bile salt chemistry by pointing out that there has been progression from  $\text{C}_{26}\text{So}$  alcohol sulfates to

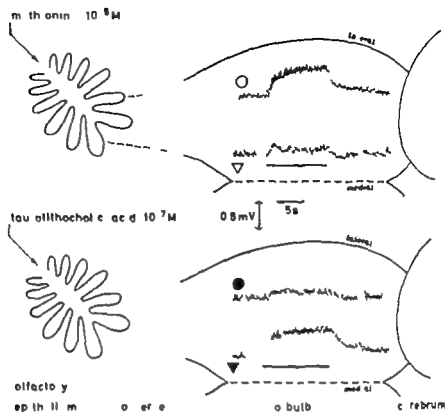


Fig. 5. The appearance of simultaneous recordings from two positions on the salmonid olfactory bulb during stimulation with an amino acid versus a bile acid.

## DISCUSSION

The results of the present series of experiments show that some bile acids, especially taurine and sulfate conjugates, are potent olfactory stimuli for salmonid fishes. Previous investigations have shown that the olfactory organ of fishes is sensitive to amino acids (Sutterlin & Sutterlin 1971; Suzuki & Tucker 1971; Belghaug & Døving 1977; Hara 1973, 1976; Caprio 1978). In our present study we demonstrate that the fish olfactory system is more sensitive to some of the bile acids than it is to amino acids. Responses to the bile acids in the olfactory bulb are spatially separated from those to the amino acids; thus the results have implications for our understanding of the discriminatory mechanisms in the fish olfactory system. Bile acids and their derivatives have chemical properties that render them interesting as mediators of information in the aquatic environment. The possibility that these substances might be important for many functions in the life span of fishes, especially in the homeward migration of salmonids, is of particular concern. In the following, different aspects of the function of the fish olfactory system will be considered in view of this new information.

**Sensitivity.** The amino acids have previously been considered to be the most potent group of olfactory stimuli for fish. In this study, bile acids as stimuli, responses were obtained from olfactory bulb surface at much lower concentrations than ever observed when using amino acids. The bile acid derivatives are therefore presumably more potent as olfactory stimuli than the amino acids or any other group of substances examined by electrophysiological techniques (Hara 1976; Belghaug & Døving 1977).

The bile acids may be suspected of having locally irritating effects on receptor membranes because of their strong detergent properties. However, the substances did not evoke activity in areas responding to amino acids as they would have done if their effect had been a general stimulation of all receptors. This high sensitivity to the bile acids can only be explained as a specific sensitivity to this particular molecular configuration. If the olfactory receptors there must be acceptors specifically designed to react to the bile acid derivatives.

**Spatial distribution.** Thommesen (1978) shows that stimulation of the olfactory organ of salmonids

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C<sub>15</sub>β acids. There are unique stages in the vertebrate evolution that might be considered representative for the different vertebrate groups. Thus the bile salt history could indicate that a concomitant sensitivity to these chemical substances has a survival value. The known variability of bile salts and the multitude of their derivatives of which only a minor part is known, yield interesting perspectives for chemical communication among fishes. We demonstrate in the present experiments that the fish olfactory system is sensitive to some of these compounds, that it is similarly sensitive to bile per se and that there are substances of bile salt character in the water inducing olfactory response in the same areas of the bulb as the pure bile compounds. These observations all indicate that the bile and bile derivatives might be valuable sources of information for fishes.

**Bile salts properties.** The principle function of the bile salts in the vertebrate body is to aid in the digestion of food lipids by their detergent properties. Their chemical properties make them particularly interesting in the aquatic environment because some of them are readily degraded and would have only a short term effect on a fish. Others are more stable and are absorbed by organic matter and minerals in the water. This could give the fishes a possibility for recognizing their own territory.

**Homing and olfaction.** The homing of salmonid fishes has been shown to depend upon the intact function of the olfactory organ, as for example demonstrated by Wisby & Hasler (1954) and Groves et al. (1968). Nordeng (1971, 1977) has on the basis of extensive field observations proposed that anadromous salmonids follow odour trails which are produced by other individuals of the same population in the water system. To secure a successful homing based on olfactory cues the threshold of the olfactory system of each individual migrating fish needs to be lower than the concentration of odorants emanating from the fish population in the water system. This reasonable assumption can be formulated to yield a general expression of the possibility of fishes to home securely by olfactory cues. The ratio between the concentration of fish odorants in the water system and the odour threshold in homing fish expresses a safety factor for homing. The concentration will depend upon the number of fishes in the river, the production of odorous substances from each individual fish and the flow of water through the system.

This study was supported by the Norwegian Fish Research Council. We wish to thank B. Sæbø, N. Midvedt for supplying most of the bile acid samples. We also wish to thank O. A. H. Hildebrand for advice.

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# Effect of a pressure barrier on retrograde axoplasmic transport in vitro. A study in the motor neurons of the rabbit vagus

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Retrograde axoplasmic transport, demonstrated by the double isotope method was studied in vitro in the motor neurons of rabbit vagus nerve, labelled with  $^3\text{H}$ -leucine. When pressure barrier with maximal pressure of 30 mmHg, induced by fluid jet, was applied to small section of the nerve at  $30^\circ\text{C}$ , retrograde flow was partially inhibited. More radioactivity was found in front of the barrier (with respect to direction of flow) than immediately behind it. This observation differs from findings in anterograde transport at the same pressure and treated with similar technique where most radioactivity was detected immediately behind the barrier (Hahnenberger 1978).

**Key words:** Rabbits, vagus nerve, retrograde axoplasmic transport, pressure barrier.

Retrograde axoplasmic flow by which material is transported towards the perikaryon, can be inhibited by ischemia (Kristensson et al 1971) and various agents such as iodoacetate, 2,4-dinitrophenol (Edstrom & Hansson 1973) and antimitotics (McLean et al 1976, Edstrom & Hansson 1973, 1972), indicating a close similarity to anterograde transport. However whereas anterograde occurs in vivo with at least two distinctly different rates, a fast (10-100 mm/h) and a slow (1-2  $\mu\text{m/day}$ ) (Dahlström 1971), there is up till now no data for a retrograde transport rate as slow as anterograde. Retrograde transport is approximately as fast as forward flow (Kristensson et al 1971) and seems to be more sensitive to colchicine (Levi et al 1976).

In a previous paper the effect of a pressure barrier on fast anterograde axoplasmic flow in the motor neuron of the rabbit vagus nerve was reported (Hahnenberger 1978). The purpose of the present study was to investigate the effect of pressure on retrograde transport, utilizing the same technique for local compression of the nerve.

## MATERIALS AND METHODS

### *Animals and anaesthesia*

Albino rabbits of both sexes weighing between 1.8 and 4.7 kg were used. Anaesthesia was obtained by slow i.v. injection of 7-8 ml/kg of 2.5% urethane solution (w/v). Occasionally ether was added, when surgical anaesthesia was not achieved with urethane alone.

### *Labelling of the motor neurons of the vagus nerve*

40  $\mu\text{Ci}$   $^3\text{H}$ -leucine was applied in drops to the floor of the 4th ventricle. After 3.5 h the animal was killed and 2 ligatures applied to the vagus on each side: one proximal ( $L_p$ ), rostral to the ganglion nodosum and one distal ( $L_d$ ) 50 to 60 mm from  $L_p$ . For details of the procedure see Hahnenberger (1978).

### *Pressure barrier*

The same method as described previously was used. The nerve was mounted in superfusion chamber and aerated superfusion solution (composition see Hahnenberger 1978) was directed through an annular gap around the whole circumference of small section of the nerve. Gap width was 0.2 mm. A flow rate of 290 ml/min resulted in a maximal pressure of 30 mmHg and maximal pressure gradient of 100 mmHg/mm nerve centered around the gap. This pressure barrier had been found to be not far above

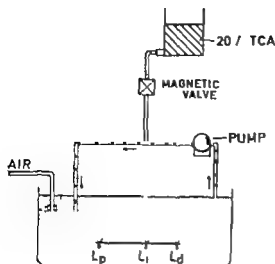


Fig 1 Arrangement for automatic interruption of axoplasmic flow

threshold for inhibition of fast anterograde axoplasmic flow at 22°C (Hahnenberger 1978). The present experiments were run at 38°C. Only one nerve of each animal was used in these experiments; the other nerve was used for other purposes.

#### Retrograde axoplasmic flow

A ligature blocks all axoplasmic transport. Anterogradely transported material soon accumulates at the ligature, but gradually some is removed (reflected) by retrograde flow away from the ligature (Bray et al 1971; Edström & Hansson 1973; Frizell 1974). A new ligature applied proximal to the first one, after the anterograde flow has passed, will then eventually collect the retrogradely moving fraction. This fraction contains components which are reflected at the distal ligature and others which have changed direction of movement en route. This technique was first described by Bray et al (1971).

After the vagus nerve was removed from the animal, the ligatures  $L_p$  and  $L_d$  applied, and the nerve cleaned. It was either incubated as described by McLean et al (1975) in tissue culture medium 199 in an oxygen atmosphere or incubated in aerated superfusion solution as shown in Fig 1. In both cases for 5 h at 38°C. Pilot studies showed that this length of time and temperature were sufficient for most of the radioactivity to accumulate in the 2 mm nerve pieces immediately proximal to  $L_d$ . There was no difference in distributions of radioactivity between nerves incubated by one or the other procedure.

After the nerves were removed from incubation a third ligature ( $L_i$ ) was placed 70 mm proximal from  $L_d$ . Most of the radioactivity subsequently collecting in the 2 mm nerve piece immediately distal to  $L_i$  thus had arrived by retrograde movement. In order to select a duration and temperature giving easily measurable radioactivities at  $L_i$  the following experiments were carried out: nerves which had been incubated for 5 h at 38°C and with  $L_d$  in place were incubated for another 5 or 1 h at 22° or 38°C.

Occasionally it was unavoidable that particular incubation experiments were scheduled to end at an inconvenient

time of night. The incubation was then interrupted by means of the system shown in Fig 1. Nerves were placed in a bath containing 100 ml superfusion solution which was circulated vigorously by a roller pump (Marlow) and bubbled with air. Above the bath was a reservoir containing 100 ml of 20% methocel solution (TCA). The reservoir was connected to the bath through a magnetic valve which was closed during incubation. At the given time 5 or 12 h after the start of the incubation the pump but not the air bubbler was switched off and the magnetic valve of the TCA reservoir was opened. This stopped any further axoplasmic movement. Some hours later the nerves were cut into segments, kept for at least 4 h in ice cold 10% TCA solution. TCA insoluble radioactivity was determined according to standard methods. The results showed that at 38°C there was sufficient radioactivity at  $L_i$  for the experiments where nerves were subjected to a pressure barrier were thus carried out at 38°C, but not automatically discontinued. The center of the nerve was placed approximately midway between  $L_i$  and  $L_d$ . At the end of the superfusion, the nerve was cut in the pressure area. The nerve segments were then cut further in 2 mm pieces under the preparation microscope and the TCA insoluble radioactivity determined.

#### Presentation of the data

Only the radioactivity in the distal nerve segment was considered to have significance for the present study and this was measured.

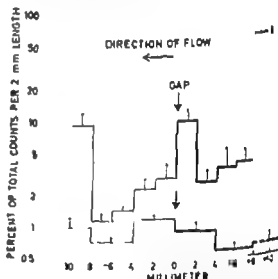


Fig. Distribution of radioactivity when retrograde (tested at 38°C for 12 h) or anterograde flow (at 22°C for 5 h) have to overcome pressure barrier of 30 mmHg. Upper graph represents retrograde, the lower one anterograde transport. The latter graph has been arranged downwards and has been constructed from data of Hahnenberger (1978, Fig. 7B). Direction of flow from right to left for both graphs. A vertical line indicates significant difference between columns ( $P < 0.05$ ) between the radioactivity is given per mm in case of retrograde and 4 mm in case of anterograde flow. Bars indicate S.E.M.

c1 Distribution of radioactivity in the distal nerve segment ( $L_1$ - $L_4$ )Figures represent mean percentages  $\pm$  S.E.M.

Location of incubation perfusion	$L_1$ (2 mm)	Intermediate (16 mm)	$L_4$ (2 mm)	Total amount of TCA-precipitable radioactivity (cpm, mean $\pm$ S.E.M.)
avg values, 0 h (5)	3.71 $\pm$ 0.76	19.3 $\pm$ 3.1	77.7 $\pm$ 2.4	5 788 $\pm$ 685
22°C (5)	4.7 $\pm$ 2.2	18.3 $\pm$ 6.6	73.4 $\pm$ 8.2	5 123 $\pm$ 427
22°C (8)	10.4 $\pm$ 2.8**	11.9 $\pm$ 4.3	78.1 $\pm$ 6.8	4 738 $\pm$ 571
38°C (n=6)	10.6 $\pm$ 3.8*	23.5 $\pm$ 6.8	65.4 $\pm$ 6.5	4993 $\pm$ 519
38°C (n=6)	18.2 $\pm$ 3.5**	34.2 $\pm$ 7.9*	47.7 $\pm$ 7.9*	5 002 $\pm$ 641
38°C (n=6) + 30 mmHg, 12 h. C (109)	9.3 $\pm$ 2.7	26.5 $\pm$ 3.1	64.2 $\pm$ 5.8	4 995 $\pm$ 713

Significance of differences against the starting values: \* $P < 0.05$  \*\* $P < 0.025$  \*\*\* $P < 0.0125$ 

the activity in each nerve piece (2 mm) between  $L_1$  and  $L_4$  is expressed as percentage of the whole amount collected between  $L_1$  and  $L_4$  (the values are given in Fig. Table 1).

be two-sample  $t$ -test or the paired  $t$ -test (when software were used to test the significance of differences).  
be expressions "proximal" and "distal" refer to the anatomical course of the vagus nerve. The direction of axonal flow is disto-proximal.

## RESULTS

ble 1 shows the distribution of radioactivity within the nerve segment  $L_1$ - $L_4$  after  $L_1$  was applied (0) and after subsequent incubation for 5 and 12 h at  $^{\circ}\text{C}$  and  $38^{\circ}\text{C}$ .

The pool of radioactivity at  $L_4$  did not differ from initial situation (0 h) when the nerves were incubated at  $22^{\circ}\text{C}$ . There was, however, an increase accumulation at  $L_1$  though only the 12 h values differed significantly from the initial values.

At  $38^{\circ}\text{C}$  the radioactivity at  $L_4$  decreased significantly with time. There was a simultaneous increase at  $L_1$ .

The column designated intermediate summarizes the radioactivity detected in the 16 mm nerve segment between  $L_1$  and  $L_4$  (16 mm = 20 mm minus the two 2 mm nerve pieces, adjacent to both nerves). This radioactivity represents mainly distal travelling between the ligatures. At  $38^{\circ}\text{C}$  and an incubation time of 12 h there was significant more radioactivity in the intermediate nerve segment than initially and after 12 h at  $22^{\circ}\text{C}$  ( $P < 0.05$ ).

Fig. 1 (upper graph) shows the distribution of radioactivity after the nerve had been superfused for 12 h at  $38^{\circ}\text{C}$ . A pressure barrier was applied

midway between  $L_3$  and  $L_4$ . Approximately 13% of the whole amount was found within  $\pm 2$  mm of the pressure area. In the 2 mm nerve pieces immediately distal to the center of the gap (in front of the gap with respect to the direction of retrograde flow) there was significantly more radioactivity than in the 2 mm immediately proximal to the centre of the gap ( $P < 0.05$ ) (behind it with respect to the direction of retrograde flow). The significance was tested by the paired two-tailed  $t$ -test.

About 9% of the whole radioactivity in the distal nerve segment was found at  $L_1$ . This amount is significantly less ( $P < 0.05$ ) than the radioactivity collecting at  $L_4$  when the nerves were run without a pressure barrier for 12 h at  $38^{\circ}\text{C}$  ( $18.2 \pm 3.5$ ).

## DISCUSSION

Retrograde axoplasmic flow can be demonstrated by two different methods. Krastensen & Ohlsson (1971) and later La Vañ (1972) introduced the already classical "protein tracer technique" in which the uptake and retrograde movement of exogenous proteins (e.g. horseradish peroxidase) applied to the periphery of axons is followed. Though this method furnishes an excellent tool which does not require damage of the neuron it is at present a semiquantitative method. The double ligature method" on the other hand provides a good quantitative resolution. However as pointed out by Prineas (1974) part of the retrograde flow could be caused by the axonal injury at the ligature rather than representing "normal" retrograde transport. Although one cannot unreservedly regard the retrograde flow studied in the present experiments as physiological, it is undoubtedly due to an active

temperature dependent retrograde transport system

Though the situation in the distal nerve segment ( $L_1$ – $L_4$ ) is too complicated to interpret completely there is one point that merits discussion. Nerves incubated for 5 and 12 h at 22° and 38°C without a pressure barrier showed the expected increase of radioactivity at  $L_1$  representing retrogradely moving material. However, only when nerves were run at 38°C was there a considerable decrease of radioactivity at  $L_4$  with simultaneous increase at  $L_1$  and in the intermediate nerve segment. This last activity may represent a slower phase of retrograde transport the existence of which has been suggested by McLean et al (1976) but the observation may also be due to a temperature dependent delay of reversal of fast moving components at  $L_4$ . The matter cannot be decided at present.

The maximal pressure of 30 mmHg in the barrier used in the present experiments was chosen for two reasons. 30 mmHg is not too far above the threshold for inhibition of fast anterograde transport and 30 mmHg has obvious relevance in connection with chronic simple glaucoma. The present results show that also retrograde axoplasmic flow is clearly inhibited by a pressure of 30 mmHg. It is difficult to judge however if the pressure sensitivity really differs from that of anterograde transport since the two forms of axoplasmic transport were tested in differently designed experiments. Anterograde flow was tested at 22°C within a nerve segment of 40 to 60 mm and run 17 h. Retrograde transport on the other hand was studied in a 20 mm nerve segment for 12 h at 38°C. In addition the anterograde axoplasmic flow studied was almost exclusively confined to the fastest travelling components while the retrograde flow was less clearly defined and may consist of different components and rates. Moreover at the starting point when the pressure barrier was applied there was presumably no radioactivity within the pressure area when anterograde flow was tested. This was different in case of retrograde flow where the compression was applied to a nerve segment already containing radioactivity.

The possibility that retrograde flow is more vulnerable is not disproved however and can derive some support from the findings of McLean et al (1976) who showed retrograde flow to be more sensitive to colchicine.

In Fig. 2 the radioactivity in the nerve pieces (4

mm each) around the centre of the gap for anterograde flow is plotted in the lower graph, to be compared with the distribution of radioactivity. Retrograde flow was tested (upper graph) reasons of clarity both the directions of flow shown from right to left and the lower graph arbitrarily displaced downwards. The figure shows the characteristic difference between the block of anterograde and retrograde flows. While anterogradely transported material accumulates immediately behind the centre of the gap (it is also supposedly the centre of the premaximum) retrograde accumulation is mainly immediately in front of the gap. The results of retrograde flow inhibition are as might be expected: there is stasis of transported material in front of obstruction. In contrast, the distribution of radioactivity in the case of anterograde transport is difficult to understand. It seems possible that accumulation in the nerve piece immediately behind the gap is an accumulation of retrograde flow. Material may have passed the hindrance in a forward direction and then reversed its flow, en route moving retrogradely at the barrier. This explanation (Hahnenberger 1978) gets support from the present data that retrograde transport is indeed accumulated in front of the impediment. But whether explanation holds quantitatively remains to be tested.

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# Control of tissue environment during vital microscopy of the microcirculation in the tenuissimus in cat

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AMUNDSON B, BAGGE U & HALJAMÄE H. Control of tissue environment during vital microscopy of the microcirculation in the m. tenuissimus in cat. *Acta Physiol Scand* 1980, 108, 139-146. Received 11 April 1979. ISSN 0001-6772. Departments of Anatomy (Laboratory of Experimental Biology), Histology and Anesthesiology University of Göteborg, Sweden.

The physiological preservation of the tenuissimus muscle preparation in cat during vital microscopy of the microcirculation is assessed, comparing the originally described technique with a modified approach. Differences in the compared techniques include modes of dissection and transillumination, core-air exposure and monitoring procedures. The original technique involves extensive dissection, inadequate temperature control and irrigation in open air. The modified technique involves less surgery due to a new illumination system, controlled heating and a Mylar foil cover on the preparation to minimize room air influences. Temperature measurements and analyses of energy metabolism (ATP, CP, glucose, O<sub>2</sub>-P and lactate) are used as objective criteria of tissue normality. The microcirculation and metabolism are evaluated during anesthesia (α-chloralose) at rest as well as at hemorrhagic shock. In the resting state muscle temperature drops to 28°C with the irrigation technique, whereas the Mylar technique keeps the temperature at 34-35°C. Neither technique causes deviations in normal metabolism. In shock, however, the temperature in the irrigated tenuissimus muscle falls 3-9°C below deep muscle temperature and there is a significantly attenuated metabolic response to ischaemia, while the Mylar preparation follows the changes of unexposed muscle both in temperature and metabolism.

**Key words:** Skeletal muscle microcirculation, vital microscopy, hemorrhagic shock, tissue environment, energy metabolism, Mylar.

Several vital microscopic techniques for study of the microcirculation in skeletal muscle have been described (Blox 1939, Zwick & Metz 1955, Hymen & Paddao 1962, Grant 1964, Baer & Han 1967, Savage et al 1970, Baer 1973, Gray 1973). These techniques are usually designed for small animals with thin muscles, easily accessible exposure and transillumination (Myrback & Wiklund 1976). Most studies on general physiological principles in central and peripheral vascular control are, however, based on experiments in larger animals, usually dog or cat. Furthermore, large animals have a rate, pattern and tolerance of metabolism that is more similar to human conditions than that of rodents and bats. In an attempt to combine the technical advantages of small

animal preparations with the physiological advantages of the cat, Brilnemark & Eriksson (1972) developed the m. tenuissimus model. Eriksson & Lissander (1972) also reported shock studies using this model. As we continued to use the m. tenuissimus preparation for further shock studies we came to realize, however, that the model in its original form was not representative for skeletal muscle in general, because the treatment of the preparation imposed serious alterations upon the local physiological environment as compared to the unexposed muscle.

Many reports (cf. Johnson 1973) emphasize the importance of careful tissue preservation in vital microscopic studies of the microcirculation. Ideally, the tissue under observation should have the



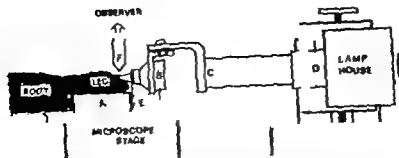


Fig. 2. Schematic drawing of horizontal illumination unit and animal preparation. A: leg dermation shelf; B: condenser with aperture diaphragm; C: extension tube; D: light-field diaphragm; E: prism holder fixed to shelf; F: objective in position above the tentacle surface.

in stripping of the tentacles muscle were eliminated. After exposure of the external surface of the muscle, a 5–10 mm long incision was made parallel to, but 1–2 mm away from the edge of the tentacles muscle in the plane extending laterally in the plane of the muscle. A pocket was then created in the loose areolar tissue underneath the muscle for introduction of the top of the prism in this way. 5–6 cm portion of the tentacles muscle immediately distal to the popliteal flex pad (Fig. 2) be transilluminated without any distortion of the light.

For the preparation the muscle was kept moist by Tyrode solution, pH 7.4 at room temperature in both horizontal groups.

**Imaging and microscopy procedures.** In both groups, animals were mounted resting on their right side on a support plate with the left leg fixed to an elevated shelf supported by a stand to ensure constant tensioners of the tentacles in all experiments. Each experimental animal had its own specially designed plate which was fixed to the cross-table of Leitz Binocular vital microscope. In the *MG* the standard illumination system of the microscope was used, while in the *MG* the light was introduced horizontally into prism and reflected on its bevel surface up through the tentacles muscle (Fig. 2).

The prism was made of plexiglass and cone-shaped with a base and 45° bevel at the tip facing a flat horizontal surface (Fig. 2) on which the muscle was positioned. A light source (Lamp House) was connected to the bevel surface of the prism. The dimensions of the prism were: base diameter 13 mm, tip diameter 6 mm and length 24 mm. The length was chosen so that the focal plane of the condenser from lens (Fig. 2) would fall immediately above the horizontal surface of the muscle preparation. The prism was held by an adjustable, vertical plate and once in position it was fixed to the shelf holding the leg. Thus the prism became immobile in relation to the tentacles muscle.

The light source was Leitz lamp house (Model 100 Z) equipped with light-field diaphragm. The condenser (Model 100, Leitz) was attached to the lamp house by a special condenser holder and metal tube, 70 cm long.

This unit was mounted on a separate stand placed beside the microscope and positioned so that the surface of the condenser front lens came in close approximation and parallel to the base of the prism. The light source unit also had to be coordinated with the movements of the microscope cross-table. To observe the entire width of the muscle belly the horizontal light beam had to be movable up and down the bevelled surface. To allow this the light source including the condenser was made vertically adjustable in relation to the prism. The light source unit was also made adjustable horizontally in the same two directions as the microscope stage to allow focal adjustments of the condenser light cone. With this system the illumination could be controlled according to Köhler principle for condenser transillumination.

For microscopy immersion objectives (Leitz) UO 11 NA 0.25 UO 23 NA 0.55 UO 35 NA 0.80 UO 75 NA 1.0 and UO 100, NA 1.00 were used.

During the experiments the microcirculation was documented from few areas of good resolution where all parts of the microcirculation could be visualized. These areas were recorded on video tape at pre-determined intervals during the control period and before, during and after bleeding.

**Local tissue environment.** In the *MG* experiments the observed tentacles muscle was exposed to room air and kept moist by intermittent irrigation with Tyrode solution, containing 2% dextran 70 at room temperature. The local temperature was recorded in the contralateral thigh musculature and selectively on the surface of the exposed tentacles muscle with a needle thermometer. In the *MG* experiments the tentacles muscle was entirely covered by a Mylar® foil soon after mounting (see Fig. 3). Mylar® is a transparent plastic film 12 µm thick which is highly impermeable to gas diffusion (Seymour 1964), thus virtually eliminating evaporation and environmental influence of room air  $PO_2$  and  $PCO_2$  on the exposed tissue. The Mylar® adhered snugly to the moist surface of the preparation and caused no disturbances to the microscopic light path.

On top of the Mylar® foil continuous drip of heated Tyrode solution was administered by peristaltic pump. The temperature of the internal tentacles muscle air

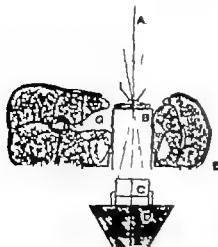


Fig. 1. Cross section of cat hind leg with glass cylinder inserted for tenuissimus muscle transillumination according to the procedure used in the irrigation group. A: objective; B: glass cylinder with tenuissimus muscle on top; C: condenser front lens; D: condenser; E: elevated shelf for the leg; F: cross-sectioned thigh muscles; G: popliteal fat pad.

same physical and chemical environment as before it was exposed. This means that it should be automatically exposed and protected from external oxygen, cooling and dilution or wash-out of local metabolic changes through external irrigation. In an attempt to better conform to these preparation requirements, Fronck & Zweifel (1975) described a modification of the tenuissimus muscle preparation. A further improvement was recently reported (1977) by the same authors. There is still a need, however, for direct metabolic data verifying the integrity of muscle tissue exposed for vital microscopy.

The present paper details an extensive development of the tenuissimus muscle model for vital microscopy where tissue environment, dissection and illumination procedures have been improved in order to obtain a better control of the preparation in terms of temperature, physiological gas tensions, interstitial fluid composition and resting state mechanics. Cellular metabolic analyses of the exposed muscle and the unexposed contralateral muscle were used for critical evaluation of the physiological preservation. To reveal and emphasize possible differences between the two sides, the energy metabolism was also provoked using a standardized hemorrhagic shock procedure. This condition also served to determine the usability of the model in shock experiments.

The material is presented in the form of a comparison of quantitative metabolic data between the original method as described by Bränek & Eriksson (1977) and the presented modified.

## MATERIAL AND METHODS

**Animals and anesthesia.** Thirty-six young female weighing 7–5 kg were used in the study. Anesthesia induced by diethyl ether and maintained by chloralose (approx. 50 mg/kg b.w.t.), supplemented with small doses (0.25–0.5 mg) of  $\alpha$ -chloral hydrate abolished twitching under the microscope. Trachea was performed to ensure free airways but respiration not assisted. A polyethylene catheter in the left jugular vein served as inlet to the central venous system. A similar catheter in the right common carotid artery connected to a Statham transducer (P 23 AC), turn was connected to a Grass polygraph for registration of systemic blood pressure. Both catheters were internally coated with heparin to prevent clotting. Body core temperature was kept at 37°C by a heater connected to a rectal thermostat.

**Dissection.** Muscle dissection procedures and subsequent mounting for microscopy were different in two groups of experiments compared in this study.

In the first group, the irrigation group (IG), the tenuissimus muscle was exposed and dissected according to the method described by Bränek & Eriksson (1977) with minor modifications as indicated below. This original procedure included a holding suture in the lateral edge of the tenuissimus muscle and a 5–6 cm long freeing of the lateral surface of the muscle from underlying structures. Considerable severing and retraction of thigh musculature beneath the tenuissimus muscle as well as lateral freeing of the muscle itself was necessary to accommodate a hollow plexiglass cone which covered the elongated condenser system. The transilluminated portion of the muscle was placed on top of this cone. In our revision of the method, the plexiglass cone was substituted by a glass cylinder 10–1 mm in diameter and with a height matching the thickness of the thigh. This cylinder was fixed on a plexiglass plate holding the leg and inserted a dissected tunnel through the medial thigh musculature that the tenuissimus muscle came to rest on its top surface (Fig. 1). With this technique, the holding suture and stretching caused by retraction were eliminated and muscle specimen could be transilluminated using a standard Leitz (Series 600) condenser. It should be noted, however, that the internal surface of the tenuissimus muscle still had to be freed from its surrounding tissue at a distance of 3 cm to allow a loose positioning on the cylinder.

In the second group of experiments, the MG group (MG), the tenuissimus muscle dissection could be significantly simplified as a direct consequence of the reconstruction of the light conducting system (Fig. 2 and 3). By medial tunneling and retraction of surrounding muscle

1. The phosphagen and glycolytic metabolite levels in the exposed and unexposed *m. tenuissimus* resting conditions and in hemorrhagic shock

Values  $\pm$  S.E. are given in  $\mu$ moles/g protein. Number of animals in parentheses. Irrigation and Mylar groups are  $\pm$  by IO and MO respectively. P values computed by Wilcoxon test.

		ATP	CrP	G-6-P	Glucose	Lactate
<i>conscious</i>						
preparation						
exposed	(4)	38.9 $\pm$ 3.7	184 $\pm$ 14.2	1.50 $\pm$ 0.33	35.4 $\pm$ 8.6	11.1 $\pm$ 3.1
unexposed	(4)	36.7 $\pm$ 3.0	185 $\pm$ 4.6	0.52 $\pm$ 0.10	20.4 $\pm$ 3.0	9.1 $\pm$ 1.2
<i>microscopy</i>						
exposed MO	(5)	34.2 $\pm$ 4.7	177 $\pm$ 24.9	1.26 $\pm$ 0.60	39.5 $\pm$ 10.3	13.5 $\pm$ 3.8
exposed IO	(5)	42.1 $\pm$ 7.0	202 $\pm$ 33.0	1.46 $\pm$ 0.96	47.3 $\pm$ 10.4	18.8 $\pm$ 6.8
unexposed	(10)	47.4 $\pm$ 4.8	211 $\pm$ 26.5	1.31 $\pm$ 0.49	26.8 $\pm$ 4.0	11.6 $\pm$ 2.4
<i>mylar shock</i>						
exposed MO	(6)	58.4 $\pm$ 7.0	47 $\pm$ 13.9	6.9 $\pm$ 1.6*	35.9 $\pm$ 8.7*	31.3 $\pm$ 5.8*
exposed IO	(7)	54.3 $\pm$ 6.5	198 $\pm$ 19.3	8.9 $\pm$ 1.7*	79.1 $\pm$ 10.9	164.8 $\pm$ 20.3
unexposed	(13)	66.7 $\pm$ 5.6	206 $\pm$ 20.2	18.7 $\pm$ 3.0	68.5 $\pm$ 7.8	117.0 $\pm$ 9.4

\* IO vs unexposed muscle.

\* MO vs unexposed muscle.

major metabolic changes occurred in the blood of animals. In both groups of bled animals, on the other hand, there were marked changes in these vital blood variables, indicating a significant shift towards anaerobic metabolism. The mean elevation of pyruvate and lactate levels was almost equal in the two groups of bled animals. On this ground it seems justified to assume that a similar metabolic situation should be present in the vital musculature of the two groups as well.

1. A collection of the metabolic analyses of *tenuissimus* muscle tissue is shown in Table 2. At the end of preparative manipulation, including mounting of *tenuissimus* muscle for microscopy, there were no significant differences in the levels of the acid metabolites between the prepared muscle and the contralateral control muscle. From a 2 h observation period in the microscope under resting lights, there were still no demonstrable differences in phosphagen or glycolytic metabolite levels

between the exposed and unexposed muscle. The tissue metabolite levels were at this point similar to those registered at the end of the preparative procedures. Furthermore, no differences in the metabolic situation between the IO and MO could be deduced from these resting condition experiments.

When muscle perfusion was compromised through bleeding, however, the levels of ATP and the glycolytic metabolites in the unexposed *tenuissimus* muscle were generally increased. These findings, which show no energy compound depletion but confirm the shift towards anaerobic metabolism, obvious from the blood samples are in agreement with our previous findings concerning early metabolic changes in skeletal muscle caused by acute hemorrhage (Amundson & Hultman 1976).

The metabolic data from the unexposed *tenuissimus* muscles of the two groups of shocked animals showed no significant differences. On the exposed side, however, the shock-induced metabolic changes

2. Rectal and muscle temperature recording after 2 h of vital microscopy under resting conditions in hemorrhagic shock.

It could be proved that the *tenuissimus* muscle temperature was externally regulated in the Mylar group (see Methods). Values  $\pm$  S.D. are given in  $^{\circ}$ C.

	Rectal temp.	Thigh muscles on unexposed side	Tenuissimus muscle on exposed side	
			Irrigation group	Mylar group
resting conditions	37	34.0 $\pm$ 0.8	28.0 $\pm$ 1.1	32-35
hemorrhagic shock	35.4 $\pm$ 1.5	29.4 $\pm$ 1.7	20.9 $\pm$ 0.2	29-31

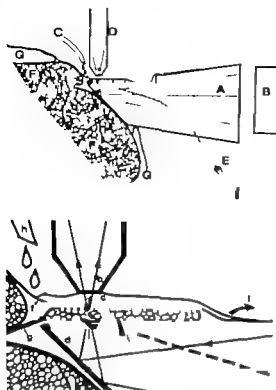


Fig. 3 Upper drawing shows prism projecting light through the tenuissimus muscle according to the Mylar model. A: prism inserted under the tenuissimus muscle. B: condenser front lens. C: fluid drip. D: objective. E: thermistor connection. F: thigh muscles. G: skin flaps. Lower drawing shows cross section of the tenuissimus muscle on prism in enlargement. a: tenuissimus muscle. b: objective. c: warming immersion fluid. d: prism tip with light reflecting bevel. e: thermistor. f: Mylar cover. g: medial attachment fascia. h: drip spout. i: drainage of immersion fluid.

face in the observation area was measured by a small thermistor attached to the tip of the prism. The temperature of the deep musculature of the contralateral thigh was monitored by a needle thermistor. The Tyrode drip was adjusted to keep the temperature in the observation area identical with that of the contralateral side. It should be noted that the Tyrode fluid never came in direct contact with the exposed tissue.

**Experimental protocol.** To evaluate the cellular effects of the preparative procedure alone, a first set of experiments was ended immediately after mounting by taking tenuissimus muscle biopsies for metabolic analyses from the exposed as well as the unexposed side. Central venous blood was drawn at the same time for pyruvate and lactate determinations. In a second set of expts., the same muscle and blood samples were taken after 2 hours of intravital microscopic observations under resting conditions. In a third set of expts., a hypovolemic state was induced once the preparation had stabilized in the microscope for 20–30 min. Hypovolemia was achieved by rapid withdrawal of 45% of the estimated blood volume (6% of b.wt.) through the arterial catheter. The resultant shock state was studied for 2 h without any additional manipulation of blood vol-

Table 1 Blood levels of pyruvate and lactate ( $\text{mmol/l} \pm \text{S.E.}$ ) in control animals and in the different shock groups

Number of animals in parenthesis. Irrigation and shock groups are indicated by IG and MG respectively

	Pyruvate	Lactate
<i>Resting conditions</i>		
End of preparation	(7) $0.074 \pm 0.018$	(17) $0.88 \pm 0.05$
2 h microscopy	(4) $0.063 \pm 0.011$	(9) $0.62 \pm 0.03$
<i>Hemorrhagic shock</i>		
2 h IG	(5) $0.285 \pm 0.034$	(5) $11.0 \pm 0.5$
2 h MG	(7) $0.268 \pm 0.04$	(7) $11.6 \pm 0.4$

ume. The experiments were terminated after the sampling procedures as described above.

In the first set of expts., only the irrigation model was used since results indicated good preservation of tissue. In the second and third sets of expts., two separate series with each model were performed. In each series, data from the exposed tenuissimus muscle and from the unexposed side were evaluated. The data from each series were combined regardless of preparation model and used for reference.

**Sample processing.** The muscle samples (0.5 g wet wt.) were immediately immersed in liquid  $\text{N}_2$  and then until homogenization, which was performed in 1 ml of ice-cold 3 M perchloric acid. After neutralization with 800  $\mu\text{l}$  of 2 M  $\text{KHCO}_3$ , the samples were centrifuged at 1000 g for 10 min. The supernatants were taken for fluorometric enzymatic determination of glucose, G6-P, lactate, CP and ATP according to previously described techniques (Hall & Eager 1975). The pellets were used for protein or nucleic acid analysis, which served as a reference standard for the metabolite levels expressed as pmol/mg wet wt.

In the blood samples pyruvate and lactate levels were determined using Sigma kits (726-UV and 826-UV).

## RESULTS

In cats not subjected to hemorrhage, a stable and largely unchanged blood pressure level was observed throughout the experimental period. The hemorrhage resulted in an initial drop in the mean arterial blood pressure to 50–60 mmHg, followed by a compensatory regain to 80–90 mmHg after 30–60 min. Towards the end of the 2 h period the blood pressure often exhibited a minor gradual decline (Amundson & Haljame 1976).

Blood levels of pyruvate and lactate of control animals and animals subjected to hemorrhagic shock are shown in Table 1. These data show that

In exposed and unexposed muscle or between the two preparative models could be detected. Despite this resemblance in metabolic the two models had very different microvascular flow patterns. In the irrigation model was a brisk, apparently hyperemic flow while microcirculation in the Mylar group was characterized by intermittency and generally slower flow rates. The hyperemia in the irrigation model readily be explained by high metabolic demands due to the low temperature in this preparation; these should be reduced compared to the Mylar group. We interpret the hyperemic reaction in irrigation model as part of an inflammatory reaction elicited by substances released by the severe preparative trauma involved in this model. In contrast, the much reduced preparative trauma

Mylar group results in an undisturbed microcirculation which probably corresponds better to metabolic demands of the tissue. A further point of good physiological preservation in the group is the fact that only with this model we been able to obtain vasoconstriction and reduction of flow upon exposure of the muscle to low oxygen tension (cf Arfors et al. 1975 and 1974, Prewitz & Johnson 1976).

In shock the hemodynamic effects of vasoconstriction, capillary occlusion and lar stasis cause the balanced nutritive tissue flow to deteriorate and be substituted by a flow distribution with a large number of occluded capillaries. These changes induce severe changes in parenchymal cellular function. The tissue metabolism is drastically altered due to local and local metabolite accumulation causes

in the irrigation model it can be suspected that irrigation fluid diluted and cleared away the metabolites since only discrete changes could be detected in the exposed muscle. On the other hand, it must be questioned to what extent such metabolic changes ever occurred in the exposed muscle since the temperature drop kept the bottom on a low level, and external oxygen have reached the muscle fibers through the irrigation fluid and maintained an aerobic metabolism in spite of a drastically reduced blood flow. Microscopic observations under such conditions become unreliable and would poorly reflect the general status of muscle microcirculation, preventing these externally mediated altera-

tions in exposed tissue composition and metabolism, the Mylar foil becomes increasingly important under shock conditions. Since it is impermeable to gases and adheres closely to the surface of the muscle only a very thin film of fluid excluded from contact with room air is formed outside the muscle. This minute volume can equilibrate with the tissue interstitium and a true *milieu interieur* representative for the shock-induced changes is contained. Tissue temperature is controlled by the external drip which cannot affect tissue oxygen tension or interstitial composition.

In the Mylar model the metabolic alterations were similar in the unexposed and exposed tenuissimus muscle also in hemorrhagic shock. We take these results as strong evidence of a representative tissue environment in the exteriorized muscle. Consequently the Mylar preparation seems to safeguard the tissue from artifactual external influences and offer a reasonable physiological environment, which make microvascular flow analyses relevant and conclusive for microcirculatory events in skeletal muscle as a whole.

The authors wish to thank Mrs Karin Almqvist and M Vojtech Sefek for excellent technical assistance.

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differed considerably between the two preparation procedures. When irrigation was used all glycolytic changes were less pronounced and there was a statistically significant difference between the exposed and unexposed muscle. The Mylar technique reduced these metabolic differences considerably between the exposed and unexposed muscle. A significant difference remained only in G6-P levels at the end of the 2 h shock period. None of the exposure techniques affected the phosphagen content of the exposed tissue to any significant degree, however.

The muscle exposure and shock procedures also resulted in temperature changes as shown in Table 3. Body core temperature decreased slightly in response to shock in spite of unchanged heating conditions. As regards the muscle recordings it should be remembered that the temperature of the exposed muscle in the Mylar group was continually controlled by the Tyrode drip to match the muscle temperature of the opposite unexposed leg.

## DISCUSSION

**Microscopy.** The quality of the vital microscopic picture obtained with the horizontal light conducting system is equivalent to that obtained with the original method for transillumination of the tenuissimus muscle (Brånemark & Eriksson 1972). The retained power of resolution is primarily achieved by maintaining a condenser system in the light unit which allows convergent light to pass through the muscle preparation. A fiber optic illumination of the tenuissimus muscle which is described and presently used by several investigators (Fronek & Zweifach 1975; Tuma et al. 1975) can be made more flexible, delicate and convenient but its emitted parallel light does not permit the same degree of resolution.

The illumination aperture of the present light source as determined by the condenser front lens is at the present 0.45 but it may be increased by the use of a shorter prism matching the Leitz 0.60/L11 condenser front lens. An even higher numerical aperture might be obtained using a light pipe system with focusing lenses e.g. something of the nature described by Starr & Netto (1973). Condensing lens systems at the tip of a light pipe are however more space demanding than the tapered prism. Such a system would therefore impose more tension and retraction in the tenuissimus muscle preparation. It

would also have to be moved in relation to the muscle when changing observation fields.

**External environment.** It has recently become evident that exteriorized tissues prepared for microscopy can be seriously influenced by microcirculatory flow characteristics by factors such as temperature, oxygen tension and irrigation procedures (Duling 1974; Clark 1975; Prewitt & Johnson 1976). Since exposure is inevitable for high resolution microscopy of tissues the influence of such external factors must be minimized to create circulatory conditions representative for the tissue in its physical surroundings. Firstly the tissue specimen must be allowed to cool off because metabolism as well as vascular tone are highly temperature dependent (Grant et al. 1931-33; Heising 1968). Secondly oxygen availability must be controlled: a drastic increase in surface oxygen tension follows room air exposure of the preparation, profoundly influence the flow rate of its supplying vessels, the same vessels that of course are most accessible for intravital microscopy. Thirdly the mode in which the tissue is kept must be of importance, in particular under the experimental conditions where major changes in interstitial composition are known or anticipated to occur. Irrigation by artificial buffer salt solutions will not only dilute and attenuate such endogenous changes which in themselves play a role in vasoregulation. Thus ideally the endogenous interstitium should be prevented from evaporation without external supplementation of fluid.

We feel that the Mylar foil presents an acceptable solution to control these external influences. It efficiently excludes oxygenation, drying and interstitial dilution in the preparation. Still, if a temperature control is fully transparent and causes no discernable tissue reaction during the observation period if applied gently and smoothly.

The choice of quantitative criteria for tissue representativity may of course be debated but in our opinion certain basic metabolic and physical variables must be considered. In addition to the obligatory temperature control we have therefore chosen to study cellular energy metabolism since these reactions are affected by variations in temperature and oxygen availability as well as by local vascular phenomena.

**Control situation.** Under resting conditions no significant differences in the analysed metabolic

an exposed and unexposed muscle or be the two preparative models could be denied. Despite this resemblance in metabolic the two models had very different microcirculatory flow patterns. In the irrigation model was a brisk, apparently hyperemic flow while microcirculation in the Mylar group was characterized by intermittency and generally slower flow rates. The hyperemia in the irrigation model truly be explained by high metabolic demands due to the low temperature in this preparation. These should be reduced compared to the Mylar group. We interpret the hyperemic reaction in irrigation model as part of an inflammatory reaction elicited by substances released by the exposure preparative trauma involved in this model. In contrast, the much reduced preparative trauma in the Mylar group results in an undisturbed microcirculation which probably corresponds better to metabolic demands of the tissue. A further argument of good physiological preservation in the Mylar group is the fact that only with this model we have been able to obtain vasoconstriction and reduction of flow upon exposure of the muscle to air oxygen tension (cf. Arfors et al 1975 & 1974, Prew et al & Johanson 1976).

In shock, the hemodynamic effects of vasoconstriction, capillary occlusion and air status cause the balanced nutritive tissue flow to deteriorate and be substituted by a flow distribution with a large number of occluded capillaries. These changes induce severe changes in parenchymal cellular function. The tissue metabolism is drastically altered due to local and local metabolite accumulation causes (cf. Prew et al 1974).

In the irrigation model it can be suspected that irrigation fluid diluted and cleared away the metabolites since only discrete changes could be detected in the exposed muscle. On the other hand, it must be questioned to what extent such metabolic changes ever occurred in the exposed muscle since the temperature drop kept the oxygen on a low level and external oxygen has not reached the muscle fibers through the irrigation fluid and maintained an aerobic metabolism. In spite of a drastically reduced blood flow, microscopic observations under such conditions become unreliable and would poorly reflect the general status of muscle microcirculation, preventing these externally mediated altera-

tions in exposed tissue composition and metabolism, the Mylar fold becomes increasingly important under shock conditions. Since it is impermeable to gases and adheres closely to the surface of the muscle, only a very thin film of fluid excluded from contact with room air is formed outside the muscle. This minute volume can equilibrate with the tissue interstitials and a true *milieu interieur* representative for the shock-induced changes is contained. Tissue temperature is controlled by the external drip, which cannot affect tissue oxygen tension or interstitial composition.

In the Mylar model the metabolic alterations were similar in the unexposed and exposed tissues of skeletal muscle also in hemorrhage shock. We take these results as strong evidence of a representative tissue environment in the exteriorized muscle. Consequently the Mylar preparation seems to safeguard the tissue from artificial external influences and offer a reasonable physiological environment which make microvascular flow analyses relevant and conclusive for microcirculatory events in skeletal muscle as a whole.

The authors wish to thank Mrs Karin Alminger and Mr Yngve Sörfelt for excellent technical assistance.

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## relative analysis of microcirculatory and cellular metabolic events in skeletal muscle during hemorrhagic shock

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Skeletal muscle reactions to hemorrhagic shock were investigated in anesthetized cats (n = 23). The tenuissimus muscle was exposed for vital microscopy and shock was induced by single-withdrawal of 45% of the blood volume. Muscle microcirculation, energy metabolism and cell membrane potentials were followed over a 2 h period along with blood pressure, hematocrit and blood leucocyte, platelet, glucose, pyruvate and lactate contents. Bleeding readily caused complete cessation of muscle blood flow for 5-20 min, while the animal compensated the blood pressure. Reflex constriction occurred in medium-sized but not in terminal arterioles. When flow reappeared, marked maldistribution was evident in the capillary bed. Flow remained in 30-50% of the capillaries, permanently or intermittently. Leucocytes could be found lodged in many arrested capillaries and also adhering in venules in large numbers. Erythrocytes or platelet plugs were not seen in the muscle microvasculature. Glucose and G6-P contents doubled and lactate increased 5-fold in muscle tissue during shock. CP was reduced by about 25% while the ATP-level remained unchanged. Membrane potentials declined 12% in shock and the spread in potentials from adjacent fibers increased.

**Key words:** Skeletal muscle microcirculation, vital microscopy, hemorrhagic shock, energy metabolism, membrane potential, high-energy phosphagens, lactic acid.

Blood flow reduction in peripheral tissues following a large acute hemorrhage, produces local disturbances in tissue metabolism and function, as transport of oxygen and nutrients becomes inefficient to meet the tissue demands. These local changes, rather than hemodynamic events, constitute the pivotal factors in an ensuing circulatory shock condition (Zweifach & Frenkel 1951; Messmer & Sander-Plassmann 1975). To gain her understanding of the pathophysiology of shock at the cell level in peripheral tissues it thus becomes pertinent to establish the precise characteristics of nutritive flow deficiencies and how fast and what extent these affect tissue metabolism and function.

The largest tissue mass sustaining hypoperfusion

in shock is the skeletal musculature. The changes in total and nutritive blood flow occurring in striated muscle in shock have been extensively described in various experimental models (see e.g. Mellander & Lewis 1963; Gregg et al. 1971; Appelgren & Lewis 1972; Eriksson & Lissander 1972b; Hutchins et al. 1973; Dawidson et al. 1979). Likewise disturbances in muscle metabolism and function during shock have been thoroughly investigated (see e.g. Le Page 1946a, b; Lima et al. 1964; Lefer et al. 1969; Dmochowski et al. 1972; Chodry et al. 1974). Primarily due to methodological difficulties little is known, however, about the detailed correlation between microcirculatory phenomena and metabolic and functional derangements in skeletal muscle in shock. This report presents vital microscopic ob-

servations of muscle blood flow changes in hemorrhagic shock utilizing a modified microcirculatory preparation (Amundson et al 1979). The microcirculatory data were combined with analyses of tissue metabolism and simultaneous recordings of muscle fiber membrane potentials (MPs) as an index of cellular function.

## MATERIALS AND METHODS

23 female cats, weighing 1.9–3.3 kg, were used. They were anesthetized by i.v.  $\alpha$ -chloralose (approx. 50 mg/kg b.wt.) after ether induction. Diazepam (0.2–0.4 mg i.v.) was given to about 50% of the animals to abolish muscular twitching. Tracheostomy was performed but respiration was not assisted. Blood pressure was measured through a heparin coated (Olsson et al 1977) PE-catheter in the right common carotid artery connected to a Statham JAC pressure transducer recording on a Grass polygraph. An identical catheter was put into a central vein through the right external jugular vein for sampling and injections. Body temperature was kept at 37°C. Anticoagulants were not given.

**Dissection and microscopy.** The left tenuissimus muscle was exposed for vital microscopy according to a technique described in a previous report (Amundson et al 1979). In short, this technique makes high resolution observations of the microcirculation possible with reduced surgical dissection due to a horizontal illumination system with a prism transmitting convergent light through the muscle. Furthermore, an impermeable plastic cover (Mylar®) and a warming drip over the exposed muscles are used to preserve and protect the internal tissue environment.

Mixed light with a green filter was used with  $\times 55$  NA 0.84 and  $\times 100$  NA 1.00 immersion objectives for high resolution observations of cellular phenomena. Monochromatic illumination at 420 nm, which increases the contrast of the red blood cells (RBCs), was used with  $\times 11$  NA 0.25 and  $\times 3$  NA 0.55 objectives for survey and flow pattern analysis. The microcirculation was systematically observed throughout the muscle segment positioned on the prism (approx. 3  $\times$  5 mm) and a few reference areas were selected for repetitive examination and documentation on video tape. Recordings were made from these areas for a minimum period of ten minutes before, during and at one and two hours after bleeding, respectively.

Flow analyses were concentrated on distributive aspects of the nutritive perfusion. An observation field visualizing 15–70 parallel capillaries was regularly used to document capillary flow patterns before and after shock. Since pilot experiments had revealed widespread standstill of capillary blood flow in shock, the number of non-perfused capillaries, i.e. vessels with zero RBC flow velocity, was regarded to be the single most relevant variable in quantitative in the present investigation.

**Experimental protocol.** After a stabilization period of 35–60 min under controlled resting conditions in the microscope, hypovolemia was induced through gradual with-

drawal of 45% of the calculated blood volume (b.wt.). The bleeding, which was carried out by syringe aspirations, was made as rapid as each rat permitted without showing signs of cardiac arrest and could usually be performed in 5–10 min. The established hypovolemic state was studied for 1 h. A further external alteration of the blood volume.

Concomitantly with the video recordings of the circulation, resting MPs were measured in dorsal muscles and foreleg flexor muscles. The recordings were made with a modified Campbell (Campion et al 1969) described in a previous report (Jamnik et al 1977).

Prior to microscopy a resting state reference sample of metabolite levels was taken from the biceps femoris muscle adjacent to the exposed tenuissimus muscle. At the end of the shock period the tenuissimus and biceps femoris muscles were biopsied bilaterally in 7 experiments. A high degree of agreement in reaction between exposed and unexposed musculature. In the remaining 16 experiments which were used for treatment studies, the left biceps femoris muscle was biopsied through a incision at the end of the shock period.

The biopsies (30–40 mg wet wt.) were immediately immersed in liquid N<sub>2</sub> and kept there until homogenized, which was performed at 0°C in 300  $\mu$ l of 3 M KClO<sub>4</sub>. After neutralization with 800  $\mu$ l of 3 M K<sub>2</sub>CO<sub>3</sub>, samples were centrifuged and the supernatants used for fluorometric enzymatic determinations of glucose, coenzyme-6-phosphate, lactate, CP and ATP levels according to previously described techniques (Hagjama & I 1975). The pellets were used for protein concentration according to the method of Lowry et al. (1951). The determinations served as reference standards for metabolite levels expressed as  $\mu$ mol/g protein.

At the instances of muscle sampling, arterial and venous blood was drawn for determination of pyruvate and lactate concentrations. Sigma Kits were used for the analyses (14-UL, 726-UL and 10, respectively). Prior to bleeding and after 1 and 2 h shock blood samples (1–2 ml) were taken for serum arterial pH, white blood cell (WBC) and platelet count.

Significance tests were performed using the Student's *t* test.

## RESULTS

**Blood pressure, pulse and temperature response.** Fig. 1 shows the computed averages of the pressure graphs expressed in mean arterial pressure (MAP). Prebleeding MAP values were mostly in the 130–150 mmHg range and heart rate 180–220 beats/min. The bleeding caused an immediate and profound pressure drop followed by prompt regain in pressure once the withdrawal of blood was completed. In 30–45 min all animals stabilized at a MAP of 60–90 mmHg. This level

Coating procedure by AB A

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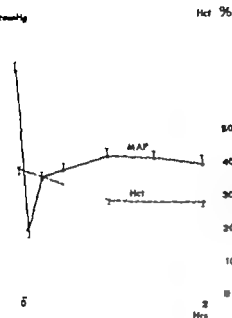


Fig. 1. Changes in mean arterial pressure (MAP) and heart rate (Hct) during the 2 h shock period. The first values of the graphs represent prebleeding mean values and values are given.

maintained over the 2 h observation period with just a slight decrease at the end in the typical case. Pulse rate, already increased by the chloralose, rose only slightly to 100 beats/min during bleeding and this level persisted through the shock period. All animals survived the initial bleeding of 45% of the blood volume for 2 h in these experiments as opposed to a 50% mortality rate in a previous study when a 50% chloralose was used under identical conditions (Anderson & Halpern 1974). With regard to the severity of the shock condition it was noted, however, that irrespective of the level of blood pressure reduction no animal seemed to tolerate further blood loss after the initial tap. This was illustrated by three animals which compensated the bleeding by maintaining MAP levels above 100 mmHg. They became unstable in blood pressure and died within a few minutes when they were rebled 5–10 ml to deepen hypotension after 30–50 mm of hypovolemia. Animals that were kept alive after the 2 h observation period without any further interventions survived for a varying length of time (range 0.5–4 h) before BP dropped in a manner similar to that of the killed animals.

In spite of unchanged heating conditions the rectal temperature dropped to 35°C after 2 h of shock,

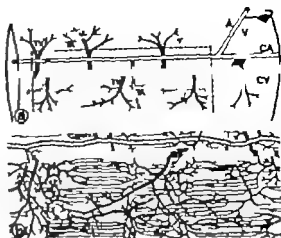


Fig. 2. (a) Principal vascular arrangement of the tenuissimus muscle. Feeding arteriole (A) and venule (V), central arteriole (CA) and venule (CV) and transverse arterioles (TA) and venules (TV). (b) Magnification of the framed area in (a) showing a semischematic drawing of the capillary bed. Only superficial vessels are included.

and the deep muscle temperature of the unexposed thigh went down from 34°C to 29–30°C during the same period. The fluid drip on the exposed tenuissimus muscle was adjusted to produce a corresponding temperature.

### Microvascular blood flow

The microvascular anatomy of the tenuissimus muscle as described by Eriksson & Myrhaug (1972) will serve as basis for the description of flow patterns (Fig. 2a). Nine categories of vessels will be discussed: Feeding arterioles and venules (average resting diameter 110  $\mu$ m and 140  $\mu$ m, respectively), central arteriole and venule (70  $\mu$ m, 90  $\mu$ m), transverse arterioles and venules (20–30  $\mu$ m, 40–60  $\mu$ m), 2–5 generations of terminal arterioles and collecting venules (8–15  $\mu$ m, 10–15  $\mu$ m) and the capillary network. Capillary dimensions have a large spread, 5.5  $\mu$ m and 1000  $\mu$ m being the means of diameter and length, respectively. All capillaries run parallel to the muscle fibers and most have 1–3 intercapillary anastomoses before reaching the venules (Fig. 2b). Dichotomous branchings and subsequent mergings within a single capillary are also regular features in this densely interconnected network. Another noteworthy observation is the consistent

appearance of tributaries from two or more terminal arterioles to a specific capillary section. Only rarely however do these tributaries stem from different transverse arterioles.

The following results are based on the analyses of approximately 40 different observation fields in 23 tenuissimus muscles studied before and at 1 and 2 h after bleeding.

**Resting state** The microcirculation in the well preserved tenuissimus muscle of the unbled resting animal was characterized by an on-and-off type of capillary perfusion with a very homogenous distribution. The intermittency of flow was extremely sensitive however to mechanical disturbances (Amundson et al. 1979). The slightest stretching or touching of the muscle would convert the on-off type of flow to a rapid continuous flow (cf. Eriksson & Myrhage 1972). The complete on-off cycle was very variable in duration ranging from 4–40 s in different expts. and the arrest period occupied 1/3 of the cycle. The flow-arrests which occurred simultaneously in all ramifications from a specific transverse arteriole clearly emanated from vasomotion in these vessels. In contrast the more distal arterioles exhibited cessation of flow without any discernable constrictions. Different transverse arterioles seemed to constrict and dilate independently of each other. As has been reported before (Lindbom & Arfors 1978) those transverse arterioles which cross the muscle and continue to arborize in the adjacent connective tissues showed less flow variations and lacked arrest periods.

During the flow phase the blood distributed to practically all capillaries with striking uniformity in flow velocity and during the no-flow phase all vessels remained open and capillaries and venules contained RBCs at all times.

The RBC capillary transit time in homogeneously perfused undisturbed muscle segments averaged 3–5 s depending upon length and width of the individual capillary. The RBCs passed the capillaries in single file with plasma gaps of 1–2 cell diameters between them. They did not form aggregates other than loose rouleaux which were mostly seen behind WBCs which usually passed the capillaries more slowly than the RBCs. These rouleaux were immediately broken up when they reached the venules.

WBCs were never a prominent feature in the resting state capillary circulation. They were most frequently found when specifically looked for in the

most distal capillaries of a given network, and that they were carried by the axial stream in arterioles. The WBCs passed the capillaries at 0–50% lower velocity than the RBCs. WBCs were not seen to block single capillaries other than occasionally in the normal state whereas a sparse adherence to the venules always occurred at all times.

**Hypovolemic state** Hypovolemia caused changes in the microvascular flow pattern of tenuissimus muscle. During bleeding, each transverse arteriole and its distal branches were used for observations of the immediate response to 1/3 of the total bleeding volume had been taken. The first wave of vasoconstriction occurred within 1–2 s after flow cessation. Typically this response was of 15–30 s duration followed by a rapid onset of As bleeding continued such an interchange between flow and no flow was evident for a few minutes. A complete circulatory arrest occurred in the muscle including the central arteriole. This period usually lasted 5–70 min. A correlation could be demonstrated between the tenuissimus muscle blood flow and the systemic blood pressure immediately after bleeding. Thus those animals which showed relatively longer cessation of muscle flow managed to compensate in blood pressure readily and to a higher level than those which had no or merely a short period of muscle flow cessation. The vasoconstriction mediated flow inhibition during and after bleeding was most pronounced in the central and even more pronounced in transverse arterioles while the more distal arterial branches did not constrict. During this period the transverse arterioles appeared as thin lines without any observable lumen while the constricted vessels looked like pale bands carrying only plasma. At the onset of recirculation small short constrictions were seen along the transverse arterioles as the solid constriction slowly gave way to flow in a wavering manner until it became continuous.

Once the flow had restarted after bleeding it was characterized by considerable inter- and intravascular variations. Distributional defects in perfusion in the form of patchiness of flow occurred on several levels. The synchronous on-and-off pattern of the resting state had always vanished in this hypovolemic shock state.

In the proximal pre-capillary vessels flow was generally continuous but showed marked variations in



Photographs of muscle capillary circulation, taken from the TV-screen. The maldistributed capillary perfusion is evident from this area, photographed before (A) and 1 h after bleeding (B). Arrows indicate areas of flow in perfused capillaries.

arterioles remained narrower during the shock period than in the unbled situation. In few transverse arterioles the early short constrictions and dilations persisted and flow was not uniform. It rarely stopped. Again in other transverse arterioles standstill of flow prevailed throughout the shock period, evidently as a consequence of prolonged constriction. This latter phenomenon, which left large areas of tissue unperfused, constituted the first level of patchiness between flow and no flow. It was prominent in some animals but insignificant or absent in most.

In the distal arterioles small flow variations could hardly be detected, plasma gaps increased in number and length and the blood failed to enter all distal branches when perfused in the resting state. Rouleaux formations became more frequent in diameter and flow rates were not significantly changed in relation to pre-bleeding values in the vessels at this level. The number of unperfused distal branches had no relation to the distance from the feeding transverse arterioles, i.e. proximal

sidebranches had a similar number of arrested terminal arterioles as distal sidebranches. In no instance was a cell or a cellular aggregate seen to block a terminal arteriole. The unperfused terminal arterioles created the second level of patchiness of flow and this was present in all transverse arteriolar networks studied.

At the capillary level the overall number of perfused vessels was usually reduced to 30–50% of all visible capillaries at any one time during the 2 h shock period (Fig. 3). In comparison the unbled resting state constantly exhibited a perfusion of about 90% of the capillaries. This capillary maldistribution which constituted the third level of patchiness in the muscle microcirculation during shock, was characterized by the absence of blood flow in a fraction of the capillaries from a certain terminal arteriole to itself continuously perfused. In such a capillary network the following observations were made:

- 1 One specific group of capillaries was constantly perfused. The linear flow rate in these exhibited a range of variation much larger than that seen in the resting state.

- 2 A second group of capillaries shifted between flow and no flow at irregular intervals, varying from seconds to several minutes in duration.

- 3 A third group of capillaries were invariably arrested when observed.

- 4 WBCs generally appeared in greatly increased numbers in the capillary bed, often impeding flow for seconds to minutes as they passed individual capillaries. During these retarded passages each WBC was always followed by a dense rouleaux of red cells, giving the cellular column the appearance of a train as it moved.

- 5 One or several WBCs were seen in a number of the permanently arrested capillaries. These WBCs, which were difficult to discover due to their very low contrast, were usually seen wedged against bulging endothelial cell nuclei along the course of a capillary or jammed in a capillary opening at a branching point.

- 6 Capillary perfusion changes concurrent with blood pressure variations occurred. Thus at the peak of blood pressure compensation one hour after bleeding a significantly larger percentage of capillaries was perfused than after two hours when blood pressure had started to decline.

- 7 Platelet or erythrocyte-platelet aggregates were never seen in the capillary network. Thus



capillary plugging by these blood constituents alone could not be demonstrated.

At the postcapillary level blood flow was substantially altered over the course of the observation period. During the initial total ischemia constriction in the central and transverse venules was prominent but it subsided as soon as a continuous flow reappeared in the arterioles. Hereby flow became continuous in the venular sections as well and during the remainder of the hypovolemic period diameter changes were not evident in any venules in comparison with prebleeding values. Instead a major feature in venules was a progressive adherence of WBCs which totally paved the venular walls in the latter part of the observation period. This uneven luminal reduction made the blood flow in a serpentine fashion between the WBCs which effectively broke up rouleaux (Fig. 4). The slow velocity was often very low but RBCs did not aggregate or attach to venular walls. Hence primary venular stoppage of flow due to cellular congestion was an uncommon finding.

Emigration of WBCs from collecting venules was frequent in late shock. Extravasation of RBCs however was rare and platelets were only observed among adhering venular WBCs. Collapse was never observed in any venular segment.

#### Membrane potentials

MP changes during the hypovolemic period are shown in Fig. 5. There was a significant decline from a resting state value of  $-90$  mV to about  $-80$

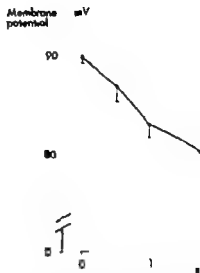


Fig. 5. Drop in muscle fiber membrane potential during the 2 h shock period. The starting point of the line represents the prebleeding mean value. SE also given.

mV after 2 h in the typical case. The decrease was linear but appeared to be more pronounced in the first hour of shock. The SE values were as the shock state progressed as an ever larger spread among measurements in the hypofused muscles. The increased spread seemed coupled to the blood pressure reaction to shock. Thus variation in MP among adjacent muscle fibers appeared more pronounced in the rats that suffered the deepest blood pressure reduction.

#### Muscle metabolism

The results of the metabolite analyses are in Tables 1 and 2. In Table 1 the tenuissimus reactions to shock are compared with those of the adjacent biceps femoris muscle. Values of the unexposed biceps femoris muscle before exposure are included as reference. Moreover the effect of exposure can be evaluated on the basis of bi-samples. Indeed these effects were slight & significant disparity only in the exposed tenuissimus G6-P content. The other analyses showed that the metabolic reactions to shock were similar in the two muscles. Once this similarity was established sampling was limited to the left biceps femoris muscle after shock. These results are presented in Table 2 as the mean total and percent difference in pre- to posthemorrhage values. The pattern of change was identical in both (Tables 1 and 2). The values were unchanged.



Fig. 4. Photograph of transverse arteriolar (A) and venular (V) segments in the tenuissimus muscle in shock taken from the TV screen. The massive adherence of WBCs (W) to the venular walls and the undulating central blood flow in the venule are typical features in shock.

1. Tissue metabolites: left biceps femoris muscle before bleeding and in right and left tenuissimus reps femoris musculi after 2 h of shock

re for microscopy was on left side where shock samples were taken from portions of the muscles covered by the od. Means from 7 expts.  $\pm$  S.E. values are given in  $\mu\text{mol/g}$  protein

#### Control samples Shock samples

Biceps femoris	Tenuissimus	Biceps femoris
Unexposed	Exposed	Unexposed Exposed Unexposed
55.2 $\pm$ 5.8	54.5 $\pm$ 5.5	58.6 $\pm$ 6.8
44 $\pm$ 8.2	196 $\pm$ 19.3	186 $\pm$ 29.6
7.5 $\pm$ 0.7	8.9 $\pm$ 1.7	17.1 $\pm$ 1.9
31.0 $\pm$ 6.0	79.1 $\pm$ 10.9	64.9 $\pm$ 12.2
21.7 $\pm$ 2.1	165 $\pm$ 20.3	130 $\pm$ 16.4
		111 $\pm$ 14.4

the CP pool was reduced by about one quarter. Glycolytic metabolites all demonstrated significant increases, which in the case of lactate was

#### 2. Mean metabolite changes in biceps femore after 2 h of hemorrhagic shock

es are expressed in concentration difference and in d of prebleeding values. Changes in blood (B) glyo- pyruvate and lactate are expressed in the same sio- E-values given. Significance level: \*\*  $P < 0.001$  vs. difference, ns = not significant

	Per cent	N of animals
$\mu\text{mol/g protein}$		
43 $\pm$ 7.4**	+5.3 $\pm$ 4.95	15
-49 $\pm$ 6.2***	25.3 $\pm$ 3.63	15
8.7 $\pm$ 1.43**	185 $\pm$ 36	15
22.9 $\pm$ 2.9***	+130 $\pm$ 23	15
69.8 $\pm$ 10.8***	665 $\pm$ 108	15
$\text{m/mole/l}$		
12.3 $\pm$ 2.1***	+111 $\pm$ 22	9
0.155 $\pm$ 0.024**	+314 $\pm$ 60	16
4.23 $\pm$ 0.81***	418 $\pm$ 94	17

#### 3. Mean blood leukocyte and platelet counts before and after 1 and 2 h of shock

two right-hand columns show mean percentage changes of prebleeding values in the shock samples. Number of /als in parentheses. S.E.-values given. Significance levels:  $P < 0.05$   $P < 0.001$  vs. zero difference, ns = not

	Before shock	1 h	2 h	% $\Delta$ 1 h	% $\Delta$ 2 h
leukocytes	9 690	7 940	17 720	+44.6**	+121
	$\pm$ 1 763 (15)	$\pm$ 1 570 (8)	$\pm$ 1 150 (15)	$\pm$ 34 (8)	$\pm$ 25 (15)
	358 000	279 000	264 000	-14.5**	-20.2*
	$\pm$ 53 300 (11)	$\pm$ 55 700 (8)	$\pm$ 34 000 (11)	$\pm$ 11.8 (8)	$\pm$ 8.4 (11)

reaction was considerable but definite consumption during shock was not seen.

## DISCUSSION

### Experimental model

The morphological resemblance between the tenuissimus muscle and thicker hind leg skeletal muscles in the cat has been documented by Myrthage (1977). The vascular arrangement of the tenuissimus muscle (Fig. 2) is found as a "basic vascular unit" in 3-dimensional form in larger muscles and its muscle fiber composition and capillary/fiber ratio are quite similar to those of other phasic muscles such as the biceps femoris and the lateral gastrocnemius muscles.

The tenuissimus muscle preparation used in this study was modified in detail to minimize the artifacts of trauma and exposure. Its vasculature has been shown to respond to changes in oxygen tension and reaction highly sensitive to mechanical trauma (cf. Lindbom et al. 1976). Furthermore the exposed muscle reacts metabolically to hypotension in a manner closely similar to that of the unexposed contralateral muscle (Amundson et al. 1979).

Using the same muscle preparation with a Saran wrap cover, Fronck & Zweifach (1977) found that one-sided exposure and microscopy for 2 h caused no change in blood flow ratio between the left and the right tenuissimus muscle when compared with the bilaterally un dissected control situation.

The slow homogeneous on-off capillary flow pattern seen in the resting state also seems to indicate an undisturbed tissue preservation. The observed mean capillary transit times (3–5 s) agree with direct measurements in the cat sartorius muscle (Burton & Johnson 1977) as well as recent morphological (Hong et al. 1977) and indicator clearance (Paaske 1977) computations. The intermittent flow characteristics have also been recently demonstrated in muscle microcirculation (Prewitt & Johnson 1976; Tuma et al. 1974). Control studies on tenuissimus muscle energy metabolism (Amundson et al. 1979) have clearly shown that this type of flow does not leave the tissue insufficiently perfused. In all probability the intermittent flow represents a normal and healthy mechanism by which capillary recruitment and volume flow are limited at rest.

On the basis of all these findings it seems justified

to assume that the present model is representative for skeletal muscle tissue and vascular control.

### Shock model

When deciding on experimental shock procedures, resemblance to clinical hemorrhage with respect to cardiovascular and hormonal reactions is a guideline. On the basis of these considerations, single withdrawal bleeding defined by shed blood was chosen as it allows the animal to benefit from a compensatory recovery in blood pressure and blood volume after complete hemorrhage. By this method the hypotension induces a sequence of reactions in the peripheral circulation which is innate to the organism and consequently similar to clinical hemorrhage (cf. Zweifach & Fronck 1977).

### Blood pressure reaction

The autonomous recovery in blood pressure evident from Fig. 1 is a clearly biphasic phenomenon. The fast increase following the postbleeding minimum is mainly the effect of reflex vasoconstriction and redistribution of blood volume (cf. Lindbom 1967). In this phase complete flow cessation prevailed in the tenuissimus muscle. The subsequent slow increase in MAP over the first hour of shock probably results from plasma volume expansion through transcapillary absorption. Early in the phase flow permanently reappeared in the tenuissimus muscle. According to Järhult's data (1977) cumulative fluid absorption from muscle amounts to 2 ml/100 g in the first hour of hemorrhage. Considering the likeness in bleeding severity to the hyperglucemia response, it is reasonable to assume a similar magnitude of absorption in our experiments. A hemodilution of that order would account for the decrease in hematocrit observed in the first hour of shock. This finding challenges the conclusion of Backström et al. (1971) who postulated sequestration of RBC aggregates in the skeletal muscle vasculature in addition to hemodilution to account for the drop in hematocrit after bleeding. However, no RBC aggregates of any sort or rouleaux could be observed in the muscle microcirculation following hemorrhage in the present study.

### Microcirculation in shock

A profound reduction in the total blood flow in skeletal muscle in response to acute hemorrhage

consistently demonstrated and accurately in whole organ preparations (see e.g. 1973). However to elucidate the cause of metabolic disturbances in hemorrhage it is essential to determine the capillary distribution. The permanent flow cessation in single vessels has the most serious impact on the tissue respect. Since this phenomenon was strikingly constant in the present experiments it was fully recorded and quantified. Evidently the effects of the nonperfused capillaries could be compensated by the flow in the capillaries perfused after bleeding (Tables 1 and 2). Apart from complete stoppage of flow the dynamic characteristics of the microvessels are appear less relevant to quantitative in relation to metabolism in shock. An emphasis on the presentation of hemodynamic variables e.g. diameter and flow velocity of the per vessels may even convey a spurious impression of uniformity inconsistent with the extreme heterogeneity of the microcirculation in shock.

In evaluating the observation of complete stoppage of muscle blood flow during and immediately after bleeding the comparatively small area of the feeding arteriole of the tenuissimus (approx. 100  $\mu$ m) and the size of the observation area (approx. 3  $\times$  3 mm) must be kept in mind.

Organ studies of muscle blood flow during hemorrhage do not report a complete cessation of flow (Lundvall & Lewné 1963, Grega et al. 1971, Järhult & Kovács 1973). Conversely these studies indicate that some flow always remains. Provided the experimental conditions of the different authors are comparable it seems reasonable to try to reconcile the whole organ data with the results on the basis of regional differences in vasoconstriction.

The theory would be that a part of the resistance vessels, the size of the longitudinal and transverse arterioles of the tenuissimus, become entirely constricted in response to the bleeding while a few arbitrarily dispersed vessels remain perfused. In a few experiments one or two perfused vessels happened to be included in the observation area, which in these cases apparently failed to exhibit a complete stoppage of flow. The reason for this incomplete coverage of the observed vasodilation during bleeding is not known to the authors.

The observed pattern of arteriolar constriction during and immediately after bleeding, which leaves

the terminal arterioles virtually uncontracted, agrees with the detailed study by Hutchins et al. (1973) in the rat cremaster muscle, and with observations in the tenuissimus muscle under sympathetic stimulation (Eriksson & Lüscher 1972a). This difference in arteriolar reaction may be due to variations in density of sympathetic innervation along the arteriolar tree or to a competitive beta adrenergic dilator response in the terminal arterioles (Lundvall & Järhult 1976). The marked dilatation of the transverse arterioles in the tenuissimus muscle soon after bleeding, reported by Eriksson & Lüscher (1972b) could not be reproduced in our experiments.

When blood flow reappeared in the muscle after the bleeding, maldistribution was evident from the start due to the reduction in perfusion pressure and greatly varying degrees of sustained vasoconstriction. In addition a very striking finding and probable cause for maldistribution was the capillary entrapment of WBCs seen in increasing numbers over the first hour of hypotension. Since this phenomenon may be of quantitative as well as qualitative importance in shock, the question of WBC plugging of capillaries was further explored in a separate study (Bagge, Arundson & Lauritzen 1979).

The unchanged WBC count after 1 h of shock (Table 3) indicates that general sequestration and mobilization of WBCs balanced over this period. After 1 h of shock mobilization dominated however concomitant with the abundant appearance of adhering WBCs in the venules. We interpret the WBC adherence and emigration as an inflammatory reaction possibly elicited by proteases released from the hypoxic muscle in shock, activating the complement system (cf. Hill & Ward 1969). Complement has been shown to increase WBC adhesiveness (Fehr & Jacob 1977, Hoover et al. 1978) and to be chemotactic for WBCs (Ward & Newman 1969).

Due to the inherent venular capacitance and the reduced volume flow rate through arteriolar constriction in the muscle in shock the WBC venular reaction did not seem to appreciably impede blood flow; however since transient increases in flow were easily conducted through the venules without signs of stasis.

Several investigators have concluded a maldistributed muscle microcirculation in hemorrhagic shock, using indirect methods (Bäckström et al. 1970, Appelgren 1972, Dahlgren 1979) as well as

vital microscopy (Eriksson & Lisander 1977b). In the Bäckström and the Eriksson reports erythrocyte aggregation and microthrombi formation are proposed to cause the capillary occlusion. In the present study neither RBC aggregates nor microthrombi were seen, possibly due to the fact that the tissue trauma was minimized in comparison with the more elaborate preparations used by other investigators (cf. Swank et al. 1964; Heideman et al. 1979).

Regardless of cause, however, it does not seem warranted to regard the heterogeneity of capillary flow as an expression of functional shunting (cf. Eriksson & Lisander 1977b; Zweifach & Fronck 1975) in the sense that blood in perfused capillaries traverses the muscle without optimal transendothelial exchange. Such a concept would require a highly increased linear flow rate in the patent capillaries, which was not seen. Thus, there seems to be no reason to suspect that the capillaries that do have flow in shock are shunting the blood without letting it perform its nutritional task in a normal way. Due to the large number of unperfused capillaries, however, multiple sections of the muscle tissue are still inadequately nourished, as indicated by the increase in anaerobic metabolism and the depressed MPs.

Although there was often a moderate decline in MAP towards the end of the shock period, no conclusive changes (i.e. dilatations) were observed in the microvascular tone. This was true also for those animals that suddenly decompensated and died within the observation period, indicating absence of arteriolar dilatation in muscle even in terminal shock (cf. Grega et al. 1971).

#### *Indices of tissue function in shock*

It is evident from the metabolic analyses that the flow disturbances observed in the microscope were severe enough to interfere with aerobic metabolism in the tenuissimus muscle. As shown in Table 1 the adjacent biceps muscle and the unexposed muscles of the other leg responded to shock in a closely similar manner. Most significantly, there was a pronounced augmentation of glycogenolytic pathways in the shock state. This resulted in a marked lactate accumulation and a gradual increase in blood lactate:pyruvate ratio. While this shift towards anaerobic metabolism took place in the tissue, the microcirculation was characterized by persistent arteriolar constriction and flow cessation in 50% or

more of its capillaries. It may be compared with Sahlin's data (Sahlin et al. 1976) on the relationship between total muscle pyruvate + pyruvate content and the lactate levels in our experiments, which would correspond to an intracellular pH of 6.6–6.8 after 7 h of shock.

The decrease in CP content also indicates a disturbance in energy metabolism. The conversion of CP to ATP may be caused by hypoxia or by a shift towards breakdown in the creatine equilibrium due to the low pH in the tissue (cf. et al. 1975). The extent of anaerobiosis seems pronounced enough, however, to affect ATP level in the resting muscle during the shock period. This finding is supported by earlier studies (Chaudry et al. 1976; Amundson & Jamlé 1976).

The decrease in MPs points to a disturbance of the normal control of fluxes and charges of the cell membrane. This is in agreement with our studies showing shock-induced disturbance of the electrolyte transport characteristics of muscle cells, resulting in a loss of intracellular potassium and an increase in interstitial pH (Hagberg et al. 1968; Haljamae 1970). The electrolyte transport disturbance seems to be related to the extent of tissue lactoacidosis rather than to the increase in tissue phosphagen content. Thus, the transmembrane charge, a complex energy-dependent phenomenon, degenerates in spite of an intact ATP content in the tissue. The exact cause of the derangement is yet unknown, although the correlation to tissue pH and lactate content points to an active role of these factors in the mechanism (cf. Jennische et al. 1978).

The more pronounced decline in MPs during the first hour of shock coincides with the large number of capillaries without flow. The observed heterogeneity in nutritive blood flow could be a factor for the increase in scatter among MPs from different fibers seen in the hypovolemic period. This conclusion is supported by earlier findings in the skeletal muscle during anaphylactic shock (Haljamae et al. 1970). The theory is that fibers farther from the vessels suffer most from hypoxia and consequently decline more in potential.

#### *In conclusion*

The skeletal musculature is subjected to profound changes in blood flow, metabolism and function during acute hemorrhage. In addition

duction in volume flow a marked capillary bottom appears. In vivo observations indicate WBC plugging is involved in this disturbance of the nutritive blood flow. Platelet or RBC aggregates are not seen in the vasculature. Adherence becomes prominent in all vessels. Aerobic metabolism is perturbed, an evident lactate accumulation and degradation content. Muscle fibers fail to uphold their potential. Precapillary vascular tone is reduced over the observation period although small animals fail to survive the shock period.

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# blood cell deformability and plugging skeletal muscle capillaries in hemorrhagic shock

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The cause of maldistributed capillary perfusion in hemorrhagic shock was investigated in a combined *in vivo* and *in vitro* study. The cat tenuissimus muscle was observed by vital microscopy before and after withdrawal of 45% of the blood volume. The induced shock conditions reduced the number of perfused capillaries about 50%. Close examination of the restricted, nonperfused capillaries showed that they remained open and almost invariably contained one or several leukocytes. These cells were usually located at the budgings of endothelial cell nuclei, and were occasionally dislodged, recirculation immediately occurred. Platelet or erythrocyte aggregates were not seen in the microvasculature in shock. To test whether the trapping of leukocytes reflected an impaired deformability of these cells in shock or was merely pressure dependent phagocytosis, deformability studies were performed on leukocytes *in vitro*. Central venous blood was drawn before and 2 h after bleeding and leukocytes were separated by sedimentation. The passage-time of single leukocytes through a glass capillary stenosis at constant driving pressures was used as an index of cell deformability. No qualitative changes in stiffness were found after shock. The distribution of cell passage-times indicated, however, that the stiffest portion of the leukocyte population was removed from the circulation in shock.

**Key words:** Leukocyte deformability, leukocyte plugging, hemorrhagic shock, skeletal muscle, vital microscopy.

Hemorrhagic shock leads to a reduced and maldistributed blood flow in skeletal muscle (Appelgren & 1972; Dahlberg 1979). Vital microscopic observations reveal that a large number of the capillaries in the skeletal muscle cease to be perfused (von & Lissander 1972). It has been suggested that the microvasculature becomes occluded by *in vivo* and *in vitro* blood cell (RBC) aggregates (Lissander et al 1971). However, recent studies in a laboratory with an improved technique for microscopic studies of the tenuissimus muscle have shown widespread cessation of capillary flow in the absence of microthrombi (Amundson et al 1979). Instead observations were made which indicate that stoppage of the capillary flow might be due to white blood cell (WBC) plugging. These

observations prompted the present investigation which was aimed at a more detailed analysis of the behaviour of the WBCs in the capillary bed in hemorrhagic shock. In addition to the *in vivo* observations a preliminary *in vitro* study was included to analyse whether the deformability of WBCs changes in shock.

## MATERIALS AND METHODS

11 female cats, weighing 1.9-2.3 kg were used—5 for *in vivo* observations and 6 as blood donors for *in vitro* experiments. All animals were anesthetized with  $\alpha$ -chloralose i.v. (50 mg/kg b.wt) after ether introduction. Tracheotomy was performed but the animals breathed spontaneously. Body temperature was kept at 37°C. In all animals the right common carotid artery was cannulated for blood pressure





Fig 1 End of glass capillary with stenosis—magnified below (inner diameter 5  $\mu$ m)

measurements and a central venous catheter was introduced through the right external jugular vein for injections and blood samples. Shock was induced by withdrawal of 45% of the estimated blood volume (6% of b wt) over a period of 5–10 min (cf. Amundson et al 1979b).

### *In vivo experiments*

The left tenuissimus muscle was exposed for vital microscopy according to a previously described technique (Amundson et al 1979a). After exposure a light conducting prism was put under the muscle and a plastic cover (Mylar®) was placed on the muscle to exclude external environmental influences.

Observations of the microcirculation were made in a Leitz Blomed microscope with water immersion objectives  $\times 23$  NA 0.55  $\times 55$  NA 0.84 and  $\times 100$  NA 1.00.

Before bleeding, representative microvascular segments in the tenuissimus muscle were selected and the microcirculation in the areas was recorded on video tape. Further, since it was known from previous experiments that many capillaries may be very difficult to discover in the vital microscope in shock due to maldistribution of the blood flow, drawings were made of the capillary network to facilitate identification of all vessels in the shock phase.

After bleeding the selected areas were reexamined at intervals for 2 h, and the number of perfused capillaries and the directions of flow were compared with the pre-bleeding recordings. Detailed observations were made on all non-perfused capillaries.

### *In vitro experiments*

An index of WBC deformability was obtained by measuring the time required for individual WBCs to deform and pass through a narrow stenosis in a glass capillary at a constant driving pressure.

Glass capillaries with a stenosis were produced according to a method described by Bagge et al (1977a) (Fig. 1). The diameter at the narrowest point of the stenosis was kept between 4.0 and 5.5  $\mu$ m. The capillaries were glued to glass slides and two basins were made of Epoxycement—one around the stenosis to contain immersion fluid for the objective and one around the distal end of the capillary for the WBC suspension. The glass slide with the capillary was placed on the stage of a Leitz vital micro-

scope which was connected to a TV-camera and recorder and a video timer (accuracy 1/100 s). Positions and video recordings of the WBC group at the stenosis were made with a water immersion  $\times 55$  NA 0.84 at room temperature (20–22°C).

The glass capillary was connected by PE-10 reservoir which could be adjusted vertically on a graduated scale for application of different hydrostatic pressures. The whole system was filled with Tyrode pH 7.4.

WBCs were obtained from blood drawn from the catheter before and after 2 h of shock. Five ml of blood was anticoagulated with 0.95 ml 15% K<sub>2</sub>EDTA. WBCs were separated from the RBCs by sedimentation in ESR tubes for one hour at room temperature.

A few drops of the WBC suspension were put at the end of the capillary and WBCs were sucked into the capillary at a constant negative pressure. The pressure was selected to obtain deformation times of the same magnitude as observed normally *in vivo* (0.5–3 s, cf. Brånnemark 1977) and ranged from 10 mm H<sub>2</sub>O to 100 mm H<sub>2</sub>O. Both samples in each experiment were tested in the same capillary using the same suction pressure.

The deformation time (DT) for conservative WBCs passing through the stenosis was measured by video analysis of the video tapes. DT was defined as the moment a WBC hit the walls of the tapered part of the stenosis and to end when the whole cell had passed the midpoint of the stenosis (Fig. 2). To determine the possible occurrence of plasma viscosity changes would influence the deformation data, particle velocities were measured for each sample at 50  $\mu$ m in a defined segment of the capillary.

Mean deformation time (MDT)  $\pm$  S.E. was calculated for all samples. In each experiment the MDT for WBCs obtained before shock was compared with the MDT for WBCs collected after 2 h of shock. To allow comparison between different experiments differences in MDT are expressed as percentage of the MDT obtained before shock.

## RESULTS

### *In vivo observations*

The rapid bleeding led to a profound drop in arterial blood pressure (MAP) from about 100 mmHg to 30–35 mmHg. When the bleeding was completed reflex compensatory mechanisms

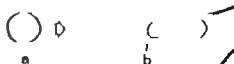


Fig. 2 The WBC deformation time required for a and b.

WBC mean deformation time (MDT)  $\pm$  S.E. before (N) and after (S) 2 h of shock in the six *in vitro*

were drawn from central aortic blood. Percentage change in MDT during shock is the right hand column. Stenosis diameter (D), deformation pressure (P) and MDP are expressed in  $\mu$ m, mm H<sub>2</sub>O and seconds, respectively. The number of cells analyzed in each sample is given in parenthesis.

D	P	MDT (N)	MDT (S)	%Δ
5.6	-30	1.95 $\pm$ 0.14 (74)	2.18 $\pm$ 0.12 (85)	+11.8
4.0	-60	1.89 $\pm$ 0.14 (50)	1.81 $\pm$ 0.13 (50)	-4.2
5.2	30	1.85 $\pm$ 0.10 (52)	1.89 $\pm$ 0.12 (49)	+2.2
5.5	30	2.00 $\pm$ 0.30 (117)	0.96 $\pm$ 0.14 (114)	-52
5.0	30	0.99 $\pm$ 0.05 (80)	0.48 $\pm$ 0.06 (65)	-51.6
5.0	10	1.16 $\pm$ 0.11 (90)	0.75 $\pm$ 0.05 (54)	-35.3

the MAP to 70–90 mmHg over a period of 30 min. After compensation all animals maintained a stable MAP throughout the observation

At the end of the bleeding a complete circulatory standstill occurred in the terminus of the aorta. This arrest period usually lasted 5–20 min after flow gradually reappeared. Capillary perfusion was evident from the start and 50% of the capillaries did not resume blood flow. The non-perfused capillaries were usually located between perfused capillaries, i.e. capillaries seen without circulation despite flow in both afferent and efferent vessels. Since these capillaries contained only a few RBCs it was only with the aid of the video tape recordings and the drawings made before bleeding that they could be de-

limited and subjected to careful observation along their entire course. In no instance were these capillaries seen to be collapsed and the RBCs could often be seen to oscillate slightly in the lumen. However a significant observation was that all of the non-perfused capillaries that were possible to observe contained one or several WBCs somewhere in the lumen (Fig. 3). Due to their low contrast the WBCs easily escaped the observer's eye at first scrutiny of the capillaries. Typically the WBCs were found lodged at the bulgings of endothelial cell nuclei or at capillary branchings (Fig. 3b). There were no indications that the WBCs were halted by adhesion to the capillary walls since they often slid smoothly back and forth in front of the hindrance. In some cases plugging WBCs were seen to be suddenly dislodged from a capillary whereupon the capillary

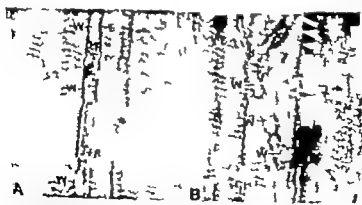


Fig. 3. In vivo photographs of WBC plugging of skeletal muscle capillaries in shock. (A) Asterisk, empty capillary (W) white blood cells, R red blood cells, E endothelial cell bulging. (B) WBC plugging at capillary branching (W) white blood cells, R red blood cells. Arrows with bars, blocked capillaries. Arrow with asterisk indicates direction of perfused flow.

was instantaneously reperused. This phenomenon clearly illustrated the plugging effect of the WBCs.

Platelet or RBC platelet aggregates were not seen in the microvasculature. Consequently capillary plugging by these blood corpuscles could not be demonstrated in the skeletal muscle microcirculation.

### *In vitro experiments*

The MAP response to bleeding in the animals used as blood donors was principally similar to that of the *in vivo* series.

The deformability data for the WBCs before and after shock are presented in Table 1. MDT values for WBCs in shock show great variation in relation to pre shock values. The mean percentage change in MDT is  $-16 \pm 9.9$  (S.E.). Statistically this figure is not significantly separated from zero when tested according to Wilcoxon's ranking test or Student's *t* test. By analysing the distribution of all DT values it seems that the scatter in MDT change is largely dependent on the number of particularly stiff cells, possibly lymphocytes (Bagge & Brånemark 1977) which happened to be present in each sample. If the stiffer cells (DT ranging from 3 to 15 s) are excluded from the calculations the MDT change becomes smaller but there is still no consistent direction of change between pre-shock and shock values.

The plasma viscosity expressed as particle velocity did not differ significantly between the samples within each experiment.

### DISCUSSION

This investigation provides evidence of WBC plugging as an important cause of capillary flow maldistribution in skeletal muscle in shock. WBC plugging of capillaries has been observed in many tissues and species including man (cf. Bagge & Brånemark 1977). However normally the plugging is only occasional and of short duration (0.5–3 s). The phenomenon is obviously related to the size, spherical shape and relatively high viscosity of the WBCs (Bagge et al. 1977a).

Passage of WBCs through the capillaries in skeletal muscle which are long and narrow (Eriksson & Myrhaug 1972; Myrhaug & Hudlická 1976) requires considerable deformations of the WBCs. In early shock, the perfusion pressure is

substantially reduced in the skeletal muscle microcirculation (Mellander & Lewis 1968). Theoretical grounds indicate that therefore the WBC plugging should occur more frequently and also be of longer duration in shock than under normal pressure conditions. Such a conclusion is supported by the present finding of frequent plugging in the tenuissimus muscle capillaries. Similar observations of WBC plugging in liver and shock have also been reported by Wilson (1977). Histological and electron microscopic studies of lungs in dogs, Wilson found a 65% increase in the number of WBCs trapped in alveolar capillaries in shock compared to controls. Further, Wilson found in both *in vivo* studies and histological sections that the WBCs tended to become larger and more spherical during shock. Theoretically such changes in the WBCs would lead to a reduced deformability and consumption of some of the membrane area available for deformation (cf. Bagge et al. 1977a; Bagge et al. 1977b). However, the present studies do not indicate that decreased WBC deformability occurs in shock. It should be emphasized that the increased frequency of WBC capillary plugging does not require a decrease in the deformability of the WBCs *per se* but can be explained by the driving pressure alone.

The redistribution of blood flow in hemorrhagic shock tends to maintain a normal perfusion of organs such as heart, brain and kidneys, while perfusion of peripheral tissues such as the skeletal muscle is markedly decreased due to vasoconstriction. The resultant reduction in capillary perfusion pressure would make the microvasculature of skeletal muscle more susceptible to WBC plugging than that of organs with a relatively higher perfusion level. In fact, histologic studies in shocked animals have failed to reveal any substantial WBC plugging in other organs than the lungs (Wilson 1977). Baeckström et al. (1971) found evidence for clogging of the skeletal muscle vasculature after a severe hemorrhage using the isolated limb preparation in cat. These investigators suggested that the plugs were formed by platelet-RBC aggregates. A normal pressure-flow relationship could be gradually restored by high pressure perfusion with a cell free medium and massage of the preparation. In our opinion such an effect could equally well be explained by WBC plugging of platelet or RBC aggregates.

The preliminary *in vitro* studies demon-

qualitative alteration of WBC deformability etc. Despite this statistical insignificance, there seems to be a tendency of the MDT to rise somewhat in shock. A tentative explanation could be that the stiffer cells within the microvasculature in a low pressure situation. To test this theory two of the present experiments were extended to include also analysis of the WBCs collected from the hind leg musculature (the deep femoral vein). The results of analysis showed a more pronounced decrease in MDT compared with pre-shock values, than WBCs from central venous blood in shock. The variation of the DT values in the samples from femoral vein indicates that the skeletal muscle vasculature is more prone to act as a filter for less deformable WBCs than other vascular circuit. This hypothesis, however, will require further investigation also involving simultaneous blood flow from the local artery and vein supplying the organ.

The observation of WBC plugging as a major cause of capillary flow maldistribution in skeletal muscle in hemorrhagic shock is striking. The experiments indicate that this is primarily a pre-related phenomenon. It can be argued, however, that the vital macroscopic observations do not reflect a generalized phenomenon since they are restricted to a small part of the musculature. The observations point to a principally major phenomenon which certainly deserves further study.

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# tid baroreflex heart rate control during the active the assisted breathing cycle in man

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The interaction between the phase of the breathing cycle and the carotid baroreflex heart rate control was studied in fifteen healthy subjects with special reference to respiratory sinus arrhythmia (RSA). Carotid baroreceptors were stimulated by neck suction. With a standardized breathing pattern, two types of experiment were performed. (A) Neck suction applied in constant phase with the respiratory arterial pressure variations, this reduced the amplitude and shifted the phase of the transmural carotid arterial pressure oscillations but did not influence the RSA. (B) During active as well as ventilator assisted breathing, brief periods of neck suction were applied during either inspiration (ventilation) or expiration. Neck suction during active inspiration did not change the amplitude of the RSA but when applied during expiration, increased it by  $6.8 \text{ beats} \times \text{min}^{-1}$  ( $P < 0.001$ ). In the ventilator experiments, neck suction during inspiration and expiration augmented the RSA equally by  $5.3 \text{ beats} \times \text{min}^{-1}$  ( $P < 0.05$ ). The results indicate a decreased sensitivity of the carotid arterial baroreflex during active inspiration, with no equivalent during assisted breathing, and suggest that the change in baroreflex sensitivity is a prerequisite for the rise of the RSA.

**Key words:** Arterial baroreflex, cardiac reflexes, heart rate regulation, intermittent positive pressure ventilation, respiration, sinus arrhythmia.

Interaction between the phases of the breathing cycle and heart rate is well known in laboratory animals and man. During quiet breathing it appears as respiratory sinus arrhythmia (RSA), i.e. an inspiratory acceleration of the heart rate and a deceleration during expiration. The arrhythmia has been attributed to several mechanisms. Early theories (for review see Hayman & Neil 1958) indicated that the RSA was mediated by a pulmonary vagal reflex activated by the expansion of the lungs during inspiration. Other animal experiments suggested that the RSA could be due to an influence from the respiratory centre (and higher centres) within the central nervous system on the cardiovascular system in the brain stem. Bambridge (1920) however suggested that the inspiratory tachycardia was mediated by a heart acceleration reflex elicited by increased diastolic filling of the heart during inspiration. Finally the systemic arterial baroreflex has

been implicated in the genesis of RSA (Matthes & Ebeling 1948; Meckelke 1953). It was claimed that the RSA was secondary to the respiratory oscillations of the arterial pressure which activated the baroreflex.

In a recent study of the central hemodynamics and RSA in man (Freyschuss & Melcher 1976) the heart rate during the breathing cycle displayed a positive correlation, beat-by-beat, to the systemic arterial pressure. One is therefore inclined to question the systemic arterial baroreflex as a cause of the arrhythmia, since this would be expected to yield a negative correlation, reflecting its depressor effect. However the baroreflex may be involved in another manner: animal experiments have demonstrated that the sensitivity of the reflex may change during the respiratory cycle (Koeppen et al. 1961; Hayman & McCloskey 1975; Davidson et al. 1976). Thus a lowered sensitivity during inspira-

tion would allow a simultaneous rise in heart rate and arterial pressure a drop in rate during expiration in spite of falling pressure could be explained by a restored reflex sensitivity. Experiments in man have supported this view (Melcher 1976 Eckberg & Orhan 1977).

It is not known what mechanisms might cause an inspiratory inhibition of the arterial baroreflex. However it has been shown that the baroreflex sensitivity can be influenced by the activity of higher nervous centres (see Smith 1974) and by the interaction with the afferent nervous inflow from cardiopulmonary receptors (Casterfors & Sjöstrand 1973 Vatner et al 1975 Kolke et al 1975 Bevegård et al 1977). The latter findings have led to the hypothesis that the increased diastolic filling of the heart during inspiration activates a cardiac reflex which inhibits the arterial baroreflex (Melcher 1976). If so inhibition would not occur during insufflation of the lungs when cardiac filling is impaired.

The present study was performed (a) to test whether or not the respiratory oscillations of the arterial pressure can induce the RSA via the carotid sinus baroreflex and (b) to investigate if there is a change in baroreflex heart rate control in phase with respiration in conscious man and whether this persists during intermittent positive pressure ventilation. Part of this work has been presented before (Melcher 1976).

## MATERIAL AND METHODS

15 healthy male volunteers participated in the study. Their mean age was 27.7 years (range 1-37). The investigation had been approved by the Ethical Committee of the Karolinska Institute.

The subjects breathed sinusoidally in pace with a beam on an oscilloscope at a rate of 6 c/min. This rate was chosen to produce an optimal sinus arrhythmia (Angelone & Coulter 1964).

The baroreceptors of the carotid artery were stimulated by applying subatmospheric pressure to the neck ( $P_{\text{neck}}$ ) thereby increasing the transmural arterial pressure (Ernsting & Parry 1957 Bevegård & Shepherd 1966). The equipment used comprised a stiff wide collar connected to a vacuum pump. The collar extended cranially in the ear lobes and was supported at its upper edge laterally by the mandible and dorsally by the processus mastoideus and occipital bone. Against the thorax it rested on the clavicles and the ventral part of the upper thoracic aperture. The edges were lined with foam rubber. The pressure within the collar was regulated by the effect of the pump. It could be varied either slowly by changing the effect of the pump or in an approximately square wave

fashion by using a stop-cock between the collar pump. The collar was equipped with one carotid transducer for monitoring of  $P_{\text{neck}}$  and another arterial pressure transducer in order to account for pressure differentially against the pressure on neck thus equalling the transmural pressure of the artery ( $P_{\text{car}}$ ). Monitoring of the esophageal pressure in the subject showed no transmittance of  $P_{\text{neck}}$  to intrathoracic pressure.

Tidal volume ( $V_T$ ) was measured by pneumography. The subjects breathed through a mouth piece the airflow was sensed by a Fleisch flow head in a differential pressure transducer (EMT 33, Servo, Sweden).  $V_T$  was obtained from the flow by electrical integration. The system was calibrated against a water sphygmometer or a precision pump (Norlander 1966) immediately before and after the experiment.  $V_T$  is given in liters ATPS.

Intermittent positive pressure ventilation (IPPV) was performed with a respirator (Servo Ventilator Siemens-Elema) with a respiratory frequency of 4 min<sup>-1</sup> and a tidal volume of 1.5 l. The ventilator specially designed so the insufflation phase could be tended to comprise 50% of the respiratory cycle end-inspiratory pressure ( $P_{\text{el}}$ ) was obtained from the titlator and recorded together with the other variables.

Intrathoracic pressure was recorded in one subject using the ordinary oesophageal balloon technique.

Heart rate (HR) was counted from ECG leads either by a heart rate meter (Ohl) Ektabrometer, or by hand from a recording (paper speed 50 mm/s).

Pressures were measured with capacitance transducers (EMT 33 and 34 Siemens-Elema, Sweden).

The variables were recorded on an ink jet recorder (Mingograf 81 Siemens-Elema, Sweden).

## Procedures

The experiments were performed with the subject in the supine position. In experiment (A) (see below) a short catheter was inserted percutaneously into a brachial artery for pressure monitoring. Before the actual experiment started intermittent neck suction of varying depth was applied in order to accustom the subjects to the new negative pressure. They also practised breathing the respirator and no measurements were begun until the subjects were able to relax and could avoid activation. IPPV recordings were accepted only if the respiratory pressure rose steadily during insufflation.

Two series of experiments were performed. (I) In subject moderate neck suction synchronized with respiration was applied sinusoidally in counter phase to the oscillation of the systolic arterial pressure to minimize and to shift the phase of the transmural pressure variations in the carotid artery (counter stimulation). Neck suction was adjusted manually in pace with subject's respiration on 5 occasions and afterwards controlled in 4 subjects. (II) In 15 subjects square neck suction was applied for approximately 3 s, either in inspiration or expiration 4 experiments. However the data were discarded since the subject spontaneously increased their  $V_T$  by more than 50%.

respiration. Thus, the results from 11 subjects are  
 1 experiments the subjects were instructed to  
 in pace with the oscilloscope beam with raised  
 breathe spontaneously chosen V (except for the  
 or expir.). In between periods of measurements the  
 were disconnected from the mouth piece

#### RESULTS

nal values of all variables were calculated as means  
 ± 6 (usually 5) consecutive breaths. The following  
 re extracted tidal volume ( $V_T$ ), maximum and  
 am heart rate ( $HR_{max}$  and  $HR_{min}$ ), amplitude of the  
 ( $HR_{max} - HR_{min}$ ), maximum and minimum carotid  
 transarterial systolic pressure ( $P_{cs}$ ) and its  
 ode ( $P_{cs}$ , only in expts (A)) the time for oc-  
 currence of these peak values during the respiratory cycle  
 -time to the start of inspiration (defined from the  
 tachograph tracing) and, finally the subatmo-  
 -spheric pressure in the neck collar  
 -sponses were performed on systolic arterial pres-  
 -sure this has shown good correlation to heart rate in  
 of the arterial baroreflex function (Smyth et al.

effects of the carotid sinus baroreceptor stimulation  
 been tested with the paired *t*-test, levels of signifi-  
 $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*)

#### ULTS

##### Effect of carotid artery counter stimulation (CS)

are summarized in Fig. 1 and Table 1. Neck  
 -ion during CS varied between 0 and -10.1  
 -g. By adjusting the order of  $P_{cs}$  to early inspi-  
 -m it was possible to reduce the ordinary res-  
 -ory  $P_{cs}$  oscillations by 27%. During control  
 -iding the  $P_{cs}$  varied between 124 and 112  
 -Hg and during CS between 127 and 119 mmHg.  
 -appears from Table 1 and Fig. 1 the occurrence  
 -maximum and minimum  $P_{cs}$  were delayed

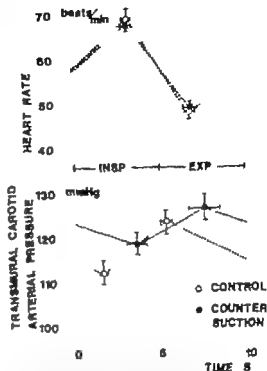


Fig. 1 Average ( $\pm$ S.E.) peak heart rates and systolic transarterial carotid arterial pressure during the breathing cycle ( $n=9$ ). Lines between peak values do not represent true time course

and the phase relation between  $P_{cs}$  and re-  
 spiration was changed. In 4 subjects the ordinary  
 pressure oscillations were almost reversed in rela-  
 tion to breathing by this stimulation pattern. The  
 RSA however was not affected neither in its  
 amplitude and peak values nor in its time course  
 during the breathing cycle (Table 1, Fig. 1)

Table 1. Effect (mean  $\pm$  S.E.) of carotid artery counter stimulation (CS) on tidal volume ( $V_T$ ), maximum sinus arrhythmia (RSA), carotid arterial transarterial systolic pressure ( $P_{cs}$ ) and time for peak rates of heart rate ( $HR$ ) and  $P_{cs}$  ( $n=9$ )

	$V_T$ (l ATPS)	Amplitude of		Time after start of step			
		RSA beats min <sup>-1</sup>	$P_{cs}$ (mmHg)	$HR_{max}$ (sec)	$HR_{min}$ (sec)	$P_{cs, max}$ (sec)	$P_{cs, min}$ (sec)
control	1.92 $\pm$ 0.13	21.1 $\pm$ 2.0	11.3 $\pm$ 0.5	3.3 $\pm$ 0.2	6.7 $\pm$ 0.3	5.3 $\pm$ 0.3	1.6 $\pm$ 0.1
Interference	1.96 $\pm$ 0.18	19.6 $\pm$ 2.3	8.2 $\pm$ 0.9	3.2 $\pm$ 0.3	6.8 $\pm$ 0.4	7.6 $\pm$ 0.9	3.6 $\pm$ 0.7
CS	0.84 $\pm$ 0.05	1.3 $\pm$ 0.6	3.1 $\pm$ 0.9	0.1 $\pm$ 0.3	0.1 $\pm$ 0.2	2.2 $\pm$ 0.7	2.0 $\pm$ 0.7
			$P < 0.01$			$P < 0.05$	$P < 0.05$



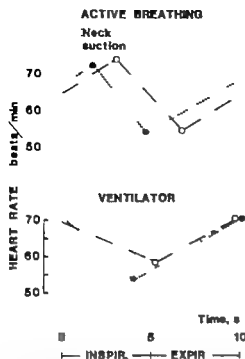


Fig. 2 Effect of inspiratory neck suction on peak values of heart rate during active and ventilator assisted breathing. Means from eleven subjects. Circles represent control and dots neck suction experiments. Lines between peak values do not represent true time course.

*The effects of inspiratory and expiratory carotid sinus baroreceptor stimulation during active and ventilator assisted breathing*

Results are presented in Figs. 2 and 3 and Table 2. During active breathing the mean  $P_{\text{atm}}$  was  $-27.3$  mmHg and in the ventilator expts it averaged  $-26.7$  mmHg. Individually identical  $P_{\text{atm}}$  were used

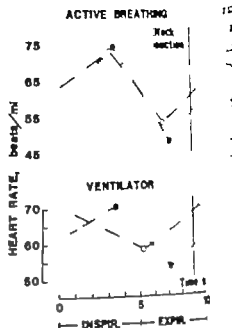


Fig. 3 Effects of expiratory neck suction on peak values of heart rate during active and ventilator assisted breathing. Means from eleven subjects. Symbols as in Fig. 2.

during inspiration and expiration stimulus averaged  $14.7$  cm of water in the ventilator or expts. It was not affected significantly by the suction.

During active breathing, inspiratory neck suction influenced neither the amplitude of the RSA nor  $HR_{\text{max}}$  or  $HR_{\text{min}}$  but peak values of heart rate late in the breathing cycle occurred significantly earlier than the ventilator control expts. with IPPV the effect appeared as a drop in heart rate during inspiration.

Table 2 Mean ( $\pm$  S.E.) control values and effects of inspiratory (IS) and expiratory (ES) baroreceptor stimulation by neck suction during active and ventilator assisted breathing ( $n=11$ )

	V (l ATPS)	RSA ampli (beats/min)	Time (s) from start of insp	
			HR <sub>max</sub>	HR <sub>min</sub>
Active breathing				
Control	1.86±0.19	19.9±2.0	3.02±0.4	6.65±0.35
IS	+0.10±0.07	-1.4±0.9	-1.36±0.28*	-2.06±0.49*
ES	-0.07±0.07	+6.8±1.4	+0.18±0.36	+0.45±0.41
Ventilator breathing				
Control	1.50±0.00	1.0±1.4	9.68±0.44	5.19±0.40
IS	0.00±0.00	+5.3±2.1	+0.4±0.44	-1.17±0.54
ES	0.00±0.00	+5.3±1.7*	-6.25±0.9*	+1.90±0.41

+ denotes later and - earlier during the respiratory cycle  
 $^*P < 0.05$   $^{**}P < 0.01$  and  $^{***}P < 0.001$

acceleration of the rate during expiration 2 and 3). Inspiratory neck suction during induced a more marked arrhythmia compared to the control (Table 7) due to a lower  $HR_{min}$  at the inspiration, while  $HR_{max}$  was not affected. As the time course of the arrhythmia changed with respiratory stimulation during IPPV.

1. Neck suction applied during expiration the active breathing was augmented (Table 8) appears from Fig. 3 this is because  $HR_{min}$  during expiration was lowered by 6.1 beats per min (0.01). The  $HR_{max}$  was not affected and the occurrence of peak heart rate values during respiratory cycle was unchanged. In the IPPV baroreceptor stimulation during expiration ended the arrhythmia, again by further decreasing minimum heart rate by 5.1 beats  $\times \text{min}^{-1}$  (0.01). There was a marked change of the heart rate course compared to the IPPV control ( $P < 0.001$  Fig. 3).

## DISCUSSION

The technique of stimulating the baroreceptors of the carotid artery by exposing the neck to reduced pressure has been used previously (Ernsting & Par 1957; Beveriged & Shepherd 1966; Thron et al. 1971). In the present study (series A) the systemic arterial pressure was measured differentially at the subatmospheric pressure to obtain a direct measure of the transmural pressure across the wall of the carotid artery. A prerequisite is how the reduced pressure is transmitted unaltered in the tissues surrounding the vessel. However this can be assumed for several reasons (Ernsting et al. 1967; Stenbrooks (1972); Coles (for review) & Olsson et al. 1974) and Eckberg (1976) used the technique extensively and found a 90% transmission of the reduced pressure in the tissue surrounding the internal jugular vein. The typical findings of Lodbrock et al. of a 64% transmission of -60 mmHg is difficult to assess as their technique is only briefly described, e.g. in measuring the transmission to the internal jugular vein. The pressure drop in the vein may be underestimated if not measured immediately at the site of neck suction before inflow of blood from other parts of the venous system has partly restored the pressure (Eckberg 1976). The only explanation for incomplete transmission would be a change in volume due to shift of blood with some disten-

tion of the tissues. With slightly reduced pressure (e.g. -10 mmHg) the change in volume is small and the compliance of the neck tissues are comparatively high, i.e. the pressure is well transmitted. Thus it seems justified to use neck suction quantitatively in the present experiments, series A. In the second part of the study series B where reduced pressures in the order of -30 mmHg are used the question of pressure transmission is not crucial since the responses to identical degree of neck suction during inspiration and expiration are compared.

The time course and the amplitude of the RSA in the present work are in accordance with previous results (Prosschuss & Melcher 1975) during both active breathing and IPPV. The completely unaffected RSA when CS changed the respiratory variations of  $P_{tra}$  shows that this arrhythmia cannot be explained by the ordinary negative feedback of the carotid baroreflex. Together with the ability of the baroreceptor stimulation to increase the RSA only when applied during expiration this clearly speaks in favour of the hypothesis that there is an inspiratory inhibition of the arterial baroreflex. This means that RSA would occur even in the absence of arterial pressure variations. Further evidence against the assumption that the baroreflex generates the RSA is found in the (B) expts. the effect of expiratory neck suction (lowered  $HR_{min}$ ) appears while the stimulus is still in progress, while there is no effect on  $HR_{max}$  which falls during inspiration showing that there is no off effect when suction is released. Consequently a rising arterial pressure during inspiration is not coupled to the slowing of the heart rate during expiration and a fall in pressure during expiration is not responsible for the tachycardia during the ensuing inspiration. The rapid appearance of the bradycardia response is in accordance with a reflex response time of less than one second (Eckberg 1976). The results of expts. (B) are broadly in accordance with those of Eckberg & Orphan (1977). However the present study seems somewhat more clear-cut in showing no effect of inspiratory stimulation on RSA amplitude, while they found some pulse interval prolongation. This difference may be due to differences in experimental design. Their subjects breathed at a higher unstandardized rate and with smaller V and the stimuli were of shorter duration, 0.6 s. Thus there was a more powerful inhibition of the baroreflex in my expts., possibly due to larger breaths. On the other hand it may be noted that even in the present

expts the inhibition was not complete since inspiratory stimulus was able to change the time course of the RSA. The ventilator expts. were undertaken to elucidate the possible interference of reflexes from cardio-pulmonary receptors and/or the central nervous respiratory drive with the arterial baroreflex. The  $P_{ei}$  were lower than during general anaesthesia (Hedenstierna & Löfström 1972) indicating that the subjects were unable to relax completely but still enough to abolish the inspiratory drop in pleural pressure. In similar expts this has been shown to impede the filling of the heart during inspiration (Freyschuss & Melcher 1976). The equal effects on HR of inspiratory and expiratory suction during IPPV show that there is in contrast to active breathing no inspiratory inhibition of the baroreflex. Thus lung expansion with 1.5 l does not depress the baroreflex function and the inspiratory inhibition is not of pulmonary origin. This is in keeping with recent canine expts which show that no inhibition of the baroreflex occurs during prolonged periods of inflation or slow insufflation of the lungs (Haymet & McCloskey 1975; Gandevia et al 1978).

Since there was a voluntary suppression of the nervous inspiratory activity during IPPV the difference from active breathing could be explained by a central nervous mechanism. The cortico-hypothalamic influence upon the baroreflex function e.g. during the "defence reaction", muscular exercise and during sleep and anaesthesia has been shown in several works (for refs. see Smith 1974). This explanation would also be in accordance with studies of the RSA in animals (Anrep et al 1936; Joels & Samueloff 1956; Levy et al 1966; Davidson et al 1976; Gandevia et al 1978) demonstrating a coupling between the central nervous respiratory drive and the RSA. In a previous study on man however no evidence was found for a central nervous mechanism generating the RSA (Freyschuss & Melcher 1975).

An alternative explanation would be that the baroreflex inhibition is associated with the diastolic filling of the heart: the classical Bainbridge effect. There is now experimental evidence that tachycardia can be induced from certain areas of the atria (for refs. see Linden 1975) and in some animal expts the elevated heart rate is explained by an inhibitory influence of cardiac afferent impulses on the arterial baroreflex (Castenfors & Sjöstrand 1973; Koike et al 1975; Vatner et al 1975). There

are observations indicating that this might also for man although the results are equivocal (Bevegård et al 1977; Takahashi 1979). In the present study inhibition of baroreflex was seen only when there were prerequisites for increased filling of the heart, i.e. during active inspiration.

In conclusion the present results show that RSA in man is independent of the respiratory chronous arterial pressure variations and the arrhythmia at least partly is due to inhibition of the arterial baroreflex heart rate control by the respiratory system. This inhibitory influence is independent of volume expansion of the lungs associated to active inspiration with mechanical return to the heart. Thus it is possibly of central nervous system or receptor origin at the heart.

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# Connective tissue of 'fast' and 'slow' skeletal muscle—effects of endurance training

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KOVANEN V SUOMINEN H & HEIKKINEN E. Connective tissue of 'fast' and 'slow' skeletal muscle in rats—effects of endurance training. *Acta Physiol Scand* 1980; 108: 173-180. Received 9 May 1979. ISSN 0001-6772. Department of Public Health, University of Jyväskylä and Department of Public Health Sciences, University of Tampere, Finland.

The connective tissue of two skeletal muscles having different contractile properties was investigated in trained and untrained rats. The subjects to be trained were put to run on a treadmill 5 days a week for 4 weeks. The 'slow' m. soleus (MS) showed higher ascorbic dehydrogenase activity but lower lactate dehydrogenase activity compared to the 'fast' m. rectus femoris (MRF). When whole muscles were taken into account, the concentrations of both hydroxyproline and hexosamines were higher for MS compared to MRF. In the middle section of MS there were more hexosamines than in that of MRF but no similar difference existed in hydroxyproline. The histochemical staining of collagen, however, suggested that there is also more internal collagen for MS as against MRF. It can be supposed that collagen of MRF and MS is differently distributed in different muscle connective tissue components. Compared to MS, the solubility of collagen was higher in MRF whereas no significant difference between the muscles existed in the prolyl hydroxylase activity. The concentrations of hydroxyproline and hexosamines or the solubility of collagen were not affected by the training given, but the activity of prolyl hydroxylase was increased in MS suggesting that the metabolism of collagen may be accelerated by physical training.

**Key words:** Collagen, glycosaminoglycans, prolyl hydroxylase, fast/slow skeletal muscle, endurance training, rat.

One of the mainstays and intensity of connective tissue research in both basic and applied areas is little known about the connective tissue of skeletal muscle, particularly as to the effects of physical exercise and training. Physical training is known to affect both the physical and chemical properties of several other connective tissues (Gibson & Gould 1973; Ingelmark 1945, 1948; Tipton 1967, 1970, 1975; Ylikö 1967, 1968). As to skeletal muscle, previous animal experiments have shown that there exists an obvious connection between the synthesis of the components of muscle connective tissue both in hyperplastic skeletal (Turto et al. 1974) and heart (Kovanen et al. 1972) muscles. According to Jablonski (1973) the activation of connective tissue cells is a important prerequisite to work-induced connective tissue growth.

The present investigation was undertaken to study the connective tissue of 'fast' and 'slow' skeletal muscles with special reference to the effects of endurance-type physical training. For this purpose the concentrations of collagen and glycosaminoglycans as well as the solubility of collagen and the activity of prolyl hydroxylase, i.e. an enzyme participating in collagen biosynthesis were determined.

## MATERIAL AND METHODS

### *Animals and training procedures*

Three-month-old male Wistar rats (n=25) were randomly assigned to test and control groups. The animals to be trained were gradually adapted to running on a treadmill. The training was performed on an 8° inclined motor-driven treadmill at speed of 4 m/min. The rats ran 5 days a week for 4 weeks, the daily exercise being per-

formed in two sessions: the first in the morning and the second in the afternoon. The training time was increased weekly from 2×10 min in the first week to 2×40 min in the last week.

Both groups were housed in 33×21×111 cm cages, two animals to each cage. All animals lived in normal laboratory conditions (humidity, temperature and the length of day were kept constant) and commercial pellet food (Hankkija, Finland) and water were given *ad libitum*.

### Muscle samples

After the training period, the trained and control rats were killed by decapitation and blood samples were taken for the determination of hemoglobin (cyanmethemoglobin method). Heart, m. rectus femoris (MRF) and m. soleus (MS) were excised. The hearts were opened, immersed several times in 0.9% NaCl to remove blood, dried slightly on a filter paper and weighed. After two days of lyophilization, the dry weights of the hearts were also determined. MRF and MS were horizontally cut into three sections of equal length. The muscles of both hind limbs were used in the analyses in order to get as large a number of observations as possible. The samples for histochemistry were immediately taken for handling as described below, whereas the other muscle samples were frozen and stored at -80°C for further analyses.

### Histochemical analyses

The histochemical analyses were performed on the middle section of the muscles. The samples for the histochemical determination of collagen were first kept in a cold (+4°C) Ringer solution and then fixed in Baker's formalin. The samples were handled routinely with the paraffin technique and stained with a modified Herovici staining procedure (see Herovici 1963).

The muscle samples for the myofibrillar ATPase were vertically embedded in OCT-compound (Tissue Tek, Ames Co.) rapidly frozen in isopentane prechilled with liquid nitrogen and stored at -80°C. Sections were cut with a cryostat microtome (Ames II) at -20°C and stained for myofibrillar ATPase (Padykula & Herman 1955). The fibers were identified as slow twitch (ST) or fast twitch (FT) on the basis of the ATPase activity as described by Gollnick et al. (1977). The results were expressed as a percentage of ST fibers.

### Biochemical analyses

**Enzymes of energy metabolism.** After thawing a sample of the middle section of muscle (about 60 mg from MRF and 20 mg from MS) was homogenized 12 times for 3 s in 50 volumes of ice-cold 10 mM K-phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 0.1% Triton X 100 by using a Potter Elvehjem homogenizer kept in an ice bath. A sample of the homogenate was diluted and the activities of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) were determined (Biochemical Test Combination, Boehringer Mannheim). The reaction rates were measured at +25°C with a Unicam SP 1800 spectrophotometer connected to a Unicam AR 5 recorder. The enzyme activities were expressed as micromoles NADH oxidized per minute per g wet weight of muscle.

**Hexosamines.** The samples of proximal and distal muscle were lyophilized, weighed, and then in 7 M HCl for 16 h at +103°C. An aliquot of the lyzate was adsorbed on a cationic exchange resin (50 II) and eluted with 1 M HCl (Boas 1959). The concentration of hexosamines (Gall & Berman 1965) was used as a measure of glycosaminoglycan in muscle.

**Hydroxyproline and nitrogen.** The residual hexosamine hydrolyzate was further hydrolyzed in 6 M HCl for 16 h at +120°C and the concentration of hydroxyproline (Kivirikko et al. 1967) and nitrogen & Zilversmit 1963) were assayed to indicate the amount of collagen and total protein, respectively.

**Solubility of collagen and total protein.** A sample of the middle section of muscle was homogenized three times in 4 ml of ice-cold 0.45 M NaCl by using a Potter Elvehjem apparatus kept in an ice bath. The homogenate was agitated in a swirling shaker for 24 h at +4°C, centrifuged at 32 000 × g for 60 min further at +4°C. The extraction procedure was repeated twice (Holt 1968). Both the sediment and combined supernatants were evaporated to dryness and hydrolyzed in 6 M HCl for 16 h at +120°C. From the hydrolyzates, the concentrations of hydroxyproline (Kivirikko et al. 1967) and nitrogen (Minari & Zilversmit 1963) were determined. The percentage of supernatant hydroxyproline from the (sediment + supernatant) hydroxyproline was taken as a measure of collagen soluble in 0.45 M NaCl. In the nitrogen analyses were used to estimate total protein.

**Prolyl hydroxylase (PH).** After thawing, a sample of the middle section of muscle (about 100 mg from MRF and 30 mg from MS) was homogenized twelve times in 30 volumes of ice-cold 0.02 M Tris-HCl buffer (pH 7.4) containing 0.1 M KCl and 0.1% Triton X 100 by using a Potter Elvehjem homogenizer kept in an ice bath. The homogenate was centrifuged at 15 000 × g for 10 min at +4°C. For the assay of PH activity, 130 µl of the supernatant fluid was incubated with <sup>14</sup>C-proline labeled collagen substrate (45 000 dpm) a gift from Prof. J. Kivirikko, 50 mM Tris-HCl buffer (pH 7.5), 2 mM ascorbic acid, 0.5 mM α-ketoglutarate, 0.5 mM FeSO<sub>4</sub> and mg/ml albumin in a final volume of 1 ml (Kivirikko & Prockop 1967). After 60 min of incubation at +37°C the samples were hydrolyzed in 6 M HCl for 16 h at +120°C and total <sup>14</sup>C-radioactivity (Prockop & Ebert 1963) as well as <sup>14</sup>C-hydroxyproline (Jens & Prockop 1966) were assayed in the hydrolyzate. The radioactivity was counted with an LKB-Wallac 8100 scintillation counter using an external standard channel ratio method for dpm calculation. The PH activity was expressed as dpm <sup>14</sup>C-hydroxyproline formed in 100 µg wet weight of muscle per mg sediment hydroxyproline as well as per mg supernatant protein. For the amount of hydroxyproline in the 15 000 × g sediment, the homogenate was hydrolyzed in 6 M HCl for 16 h at +120°C. From the hydrolyzate hydroxyproline was determined according to Kivirikko et al. (1967). The concentration of hydroxyproline was used as the measure of collagen in the muscle samples. Protein was determined in the 15 000 × g supernatant by a modified method.

## a Physical characteristics of trained and control rats

-3 II and number of observations are given

	Trained rats	Control rats	P
body weight (g)	318±15 (12)	318±15 (13)	n.s.
dry weight (g)	367±21 (12)	400±31 (13)	<0.01
wt of heart (mg)	1110±61 (12)	1009±71 (13)	<0.01
wt of heart (mg)	257±14 (17)	252±17 (13)	<0.001
wt of MRF (mg)	1037±73 (9)	1064±96 (10)	n.s.
wt of MRF (mg)	261±24 (9)	269±25 (10)	n.s.
wt of MS (mg)	134±19 (9)	127±12 (10)	n.s.
wt of MS (mg)	35±5 (9)	32±3 (10)	n.s.

nal (1951). To ensure that adequate amounts of protein were used in the determinations of the very different volumes of the 15 000 g water of the muscle homogenates were assayed as a representative.

## -4 Methods

d procedures were used in calculate means, standard errors (S.E.) and standard errors (S.E.). The statistical significance are calculated by Student's *t*-test non-correlating series.

## TLTS

7) shows some of the physical characteristics of trained and control rats. Compared to the control group the trained rats had lower final body weight but higher heart wet and dry weights. The muscle weights did not, however, differ significantly between the trained and untrained rats. The blood hemoglobin of the trained rats was  $17 \pm 1$  g/l and that of the untrained  $15.4 \pm 0.6$  g/l.

re muscle fiber composition and the activities of enzymes of energy metabolism are shown in

Table 4. The percentage number of slow twitch fibers in MS was about 90% whereas that in MRF was only 1 to 9%. The trained animals showed lower LDH activity compared to the control group the difference being significant in MRF. There were no significant differences between the trained and untrained rats in MDH activity although it tended to be higher in both muscles of the trained rats.

No histopathological alterations were observed in the specimens obtained from the trained and control animals. Generally it was noticed that collagen stained more strongly in the perifascicular space i.e. perimysium than around the individual muscle fibers i.e. endomysium. Furthermore somewhat more collagen was observed in MS than in MRF (Fig. 1).

When the whole muscles were taken into account, i.e. when the results from the proximal, middle and distal muscles were combined (Table 3) the hydroxyproline and hexosamine concentrations were significantly higher for MS compared to MRF whereas the nitrogen concentration tended to be higher for MRF. The hydroxyproline/nitrogen ratio

## a 2. Fiber composition and enzyme activities of m. rectus femoris and m. soleus in trained and control

-3 II and number of observations are given

Table	Muscle	Trained rats	Control rats	P
Rectus (7)	MRF	1.2±1.0 (6)	1.5±1.7 (6)	n.s.
	MS	91.0±5.9 (8)	88.3±4.9 (6)	n.s.
Soleus (10)	MRF	650±43 (9)	745±47 (10)	<0.001
	MS	205±45 (9)	238±30 (10)	n.s.
Soleus (10)	MRF	479±89 (9)	424±67 (10)	n.s.
	MS	752±140 (9)	674±93 (10)	n.s.

There was significant difference ( $P<0.001$ ) between MRF and MS in all variables.



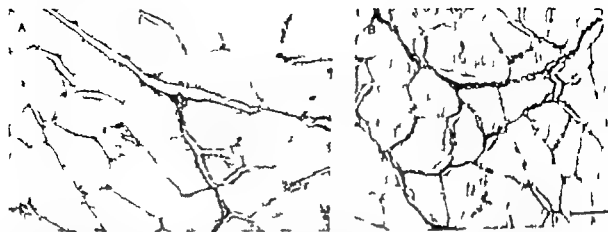


Fig. 1 Cross sections of muscle fibers from m. rectus femoris (A) and m. soleus (B) stained according to the staining procedure for collagenous connective tissue. Fibrous connective tissue of endomyrial and perimysial can be seen more clearly in m. soleus. Magnification. Bar=100  $\mu$ m

was higher in MS than in MRF but there was no significant difference between the muscles in the hexosamine/hydroxyproline ratio. There were no significant differences between the trained and control rats in respect of the above concentration values.

Table 4 shows the corresponding results on the middle sections of the muscles, i.e. the sections including the actual muscle connective tissue. There were significantly more hexosamines in the middle section of MS compared to that of MRF. In the hydroxyproline concentration there was however a reversed difference between the two muscles of the untrained animals. There was no difference between the muscles in the hydroxyproline/nitrogen ratio but the hexosamine/hydroxyproline

ratio was higher for MS compared to MRF. Percentage of soluble collagen was higher in MRF than in MS.

If the different concentration values of the different sections of the muscles are compared with the whole muscles (cf. Tables 3 and 4) lower values for hydroxyproline and hexosamines can be observed in the former muscle samples. In fact there were highly significant differences ( $P<0.01$ ) between the middle and proximal or distal sections in both muscles in the concentrations of hydroxyproline and hexosamines. The ratio of hexosamine to hydroxyproline was highest in the middle section of the muscles.

The PH activities in the two muscles of trained and control rats appear in Fig. 5.

Table 3 Biochemical properties of connective tissue of m. rectus femoris and m. soleus (whole muscles) of trained and control rats

Mean  $\pm$  S.D. and number of observations are given

Variable	Rats	MRF	MS	P
Hydroxyproline ( $\mu$ g mg d.w. <sup>-1</sup> )	Trained	9.9 $\pm$ 1.0 (9)	14.2 $\pm$ 1.6 (9)	<0.001
	Control	9.8 $\pm$ 0.7 (10)	14.1 $\pm$ 1.6 (10)	<0.001
Nitrogen ( $\mu$ g mg d.w. <sup>-1</sup> )	Trained	169 $\pm$ 18 (9)	153 $\pm$ 1 (9)	<0.05
	Control	171 $\pm$ 21 (10)	155 $\pm$ 14 (10)	n.s.
Hexosamine ( $\mu$ g mg d.w. <sup>-1</sup> )	Trained	0.87 $\pm$ 0.14 (9)	1.25 $\pm$ 0.17 (9)	<0.001
	Control	0.86 $\pm$ 0.13 (10)	1.20 $\pm$ 0.09 (10)	<0.001
Hydroxyproline/nitrogen	Trained	0.053 $\pm$ 0.007 (9)	0.093 $\pm$ 0.014 (9)	<0.001
	Control	0.059 $\pm$ 0.008 (10)	0.092 $\pm$ 0.011 (10)	<0.001
Hexosamine/hydroxyproline	Trained	0.087 $\pm$ 0.015 (9)	0.089 $\pm$ 0.014 (9)	n.s.
	Control	0.087 $\pm$ 0.015 (10)	0.086 $\pm$ 0.012 (10)	n.s.

Note: There were no significant differences between trained and control rats in any of the variables.

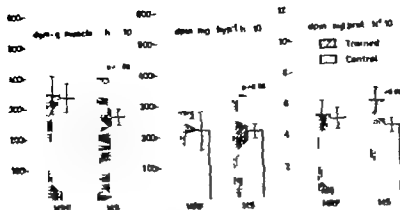


Fig. 2. p-Hydroxybenzoate activity of *m. rectus femoris* and *m. soleus* in trained and control rats. Mean  $\pm$  S.E. of 9–10 observations are given.

no significant differences between MRF and SOL. The PH activity was, however, significantly increased by training in MFS regardless of the various activities were referred to.

#### DISCUSSION

The lower body weights and the higher heart weights for the trained compared to control animals indicate that the training was effective enough to induce about the well-known physiological adaptations to endurance training. As could be expected from the results of previous results (see Holloszy 1975),

the endurance training used in this study did not, however, induce any hypertrophic growth of the two skeletal muscles.

The decreased LDH activity in MRF of the trained rats is in agreement with previous studies which show that in the muscles with high glycolytic activity the level of glycolytic enzymes decreases in response to endurance exercise (see Holloszy & Booth 1976). The MDH activity has been found to increase up to 50 to 60% following endurance training (Holloszy & Booth 1976). The training effect found in the MDH activity in the present study (10 to 15%) was not, however, statis-

#### 4. Biochemical properties of connective tissue of *m. rectus femoris* and *m. soleus* (middle section) and control rats

Mean  $\pm$  S.D. and number of observations are given.

Muscle	Rats	MRF	SOL	P
Protein (mg dry wt <sup>-1</sup> )	Trained Control	8.1 $\pm$ 1.4 (9) 7.4 $\pm$ 0.4 (10)	7.4 $\pm$ 1.3 (9) 6.3 $\pm$ 1.0 (10)	n.s. <0.05
Protein (mg dry wt <sup>-1</sup> )	Trained Control	179 $\pm$ 23 (9) 166 $\pm$ 17 (10)	139 $\pm$ 16 (9) 137 $\pm$ 13 (10)	n.s. n.s.
Protein (mg dry wt <sup>-1</sup> )	Trained Control	0.84 $\pm$ 0.14 (9) 0.79 $\pm$ 0.12 (10)	1.14 $\pm$ 0.17 (9) 1.06 $\pm$ 0.14 (10)	<0.01 <0.001
Hydroxyproline	Trained Control	0.046 $\pm$ 0.009 (9) 0.046 $\pm$ 0.005 (10)	0.047 $\pm$ 0.011 (9) 0.042 $\pm$ 0.008 (10)	n.s.
Hydroxyproline	Trained Control	0.108 $\pm$ 0.027 (9) 0.106 $\pm$ 0.016 (10)	0.139 $\pm$ 0.036 (9) 0.166 $\pm$ 0.029 (10)	<0.01 <0.001
Collagen (%)	Trained Control	4.29 $\pm$ 0.35 (6) 4.23 $\pm$ 0.97 (6)	2.38 $\pm$ 0.35 (6) 2.95 $\pm$ 0.18 (6)	<0.001 <0.05
Collagen (%)	Trained Control	30.5 $\pm$ 7.9 (6) 33.3 $\pm$ 7.9 (6)	23.8 $\pm$ 6.6 (6) 27.7 $\pm$ 5.5 (6)	n.s. n.s.

There were no significant differences between trained and control rats in any of the variables.

tically significant. The LDH activity was three times higher in MRF than in MS, whereas the MDH activity was 30 to 40% higher in MS than in MRF. This is in agreement with the earlier findings of Holloszy & Booth (1976) which showed that in the fast twitch white and red muscle fibers the activities of glycolytic enzymes are higher than in the slow twitch red fibers in which the activities of aerobic enzymes like MDH are higher. On the basis of the fiber composition the two muscles can be considered as clearly different as to their "fast and slow" contractile properties.

The collagen concentration was 40 to 50% higher in MS than in MRF when whole muscles were taken into account. The very recent findings of Laurent et al. (1978) also showed that there is considerably more collagen in the slow anterior latissimus dorsi compared to the fast posterior latissimus dorsi in the fowl. The high collagen concentrations of the tendinous distal and proximal sections of MS at least partly explain the difference between the two muscles in the present study because similar results were not observed in the middle sections of the muscles. The histochemical analyses however suggested that there may also be more internal collagen of endomysium and perimysium for MS as against MRF. The individual muscle fibers in MS were more distinct than those in MRF when stained according to the Herovici method. One reason for the observed differences between MS and MRF may be that since the ST fibers have a richer capillary supply than FT fibers (Andersen & Kroese 1978) the "slow" MS probably also has more collagen around the capillaries than the "fast" MRF. The relatively high hydroxyproline concentration as well as the low ratio of hexosamines to hydroxyproline in the middle section of MRF is apparently due to a distinct collagenous connective tissue septum which can also be macroscopically observed and which divides the muscle into two parts.

There was more collagen in soluble form (i.e. newly synthesized collagen) in MRF than in MS. It can be associated with the "slow" MS is functioning as a static position-maintaining muscle while the "fast" MRF is a more dynamic and active muscle. According to Kruggel & Fickl (1974) the solubility of collagen in an active muscle is higher than in a quiescent muscle. The endurance training did not affect the solubility of collagen in either of the muscles in the present study. This is in agree-

ment with the studies performed by Saarevaara (1978) which show that even "life-long" endurance training does not change the solubility of collagen when continually training men are compared to sedentary men.

Hexosamine concentrations were about 10% higher in MS than those in MRF when whole muscles were taken into account. The lower concentration of the middle section of MS was higher than that of MRF. This suggests that there is more ground substance, especially glycosaminoglycans in the connective tissue of MS. This notion is supported by the fact that the ratio of hexamines to hydroxyproline was also higher in the connective tissue of MS. However in agreement with Boas' (1953) assumptions, we have found in our later experiments that the Boas method gives excessive values for skeletal muscle hexosamine. Consequently the quantitative level of the results of this experiment is uncertain, but this does not influence the comparability of the results with the present study.

The training program used in this study did not induce any significant changes in the amount of connective tissue. It has also been observed that moderate endurance training does not significantly increase the concentration of hydroxyproline, hexosamines in long bones and Achilles tendon (Kuskinen 1976). This histochemical analysis (Müller (1974) indicated a local increase of connective tissue in MS, but this was obviously caused by overload during the first weeks of training. It is plausible that moderate training does not really change the concentrations of muscle connective tissue components.

The effect of training on muscle PH activity was similar to that of MDH activity, i.e. the PH activity was higher in MS than in MRF. This increase is independent of whether the enzyme activity is referred to the weight of the muscle sample or to the concentration of hydroxyproline or protein in the muscle sample. It can be assumed that the increased PH activity of MS reflects the accelerated turnover of collagen. This conception is supported by our later H-proline incorporation experiments (Kovanen et al. unpublished data) which showed that activated collagen metabolism after training according to Suominen & Heikkinen (1974) and Suominen et al. (1977) endurance training increases the PH activity of human skeletal muscle.

It can be concluded from the present observations of

that endurance training does not change the total concentrations of collagen and glycosaminoglycans in the connective tissue of skeletal muscle. The metabolism of collagen may however be accelerated. Furthermore it seems that the content of "fast and slow muscles is not equal distributed in the different muscle connective tissue components and that there are at least more staining-glycans in slow than in "fast muscles. More specific analyses on connective tissue at the level of fibers in different skeletal muscles are being performed.

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# Vasodilatation in the dental pulp produced by electrical stimulation of the inferior alveolar nerve in the cat

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The effects of nerve stimulation on blood flow were studied in the dental pulp of anesthetized cats. Changes in iodide disappearance rate ( $k$ -value) from dental cavities were used to determine changes in pulpal blood flow. Electrical stimulation of the distal end of the cat inferior alveolar nerve after  $\alpha$ -adrenoceptor blockade (phenotamine 3 mg/kg) consistently resulted in rapid increase in disappearance rate. The first stimulation produced the greatest response (an average increase in  $k$  values of 60%) and repeated stimulations showed successive attenuation in response, the fourth stimulation usually having no effect. A progressive decrease in resting  $k$ -values was observed after the first stimulation, indicating an impaired exchange function of the capillary vessels. Systemic pretreatment with propranolol (0.5-1 mg/kg), atropine (1 mg/kg), mepyramine (3 mg/kg) and cimetidine (3 mg/kg) did not influence the rapid increase in  $k$  values produced by the nerve stimulation. The experiments show that vasodilatation in the cat dental pulp produced by stimulation of the inferior alveolar nerve is not mediated by common efferent vasodilatory mechanisms and strengthens the hypothesis that the sensory nerve axon reflex mechanism is involved.

**Key words:** Dental pulp, iodide disappearance, vasodilatation, sensory nerves

Electrical stimulation of the distal stump of the second mandibular nerve in dogs has been shown to increase intrapulpal tissue pressure (Kroeger 1968) and to increase pulpal blood flow (Toonder & Naess 1974). These findings suggest that there is a neural vasodilatory mechanism in the dental pulp and raise the question of whether such neurons are functionally efferent or afferent. The presence of cholinergic fibres in the pulp may lend support to the concept of an efferent cholinergic innervation of the pulp (Pohlo & Antila 1968, Avery et al. 1974). It has also been suggested that histamine participates in the motor regulation in this tissue (Pohlo & Antila 1968, Edvall et al. 1973). Vasodilatation may also be mediated by sensory nerve fibres. Antidromic stimulation of sensory fibres has long been known to induce vasodilatation in the skin in different species (Baylis 1901, Dale & Gaskell 1930, Celander & Folkow 1953, b). This effect, which is resistant and probably mediated via pain

fibres (Celander & Folkow 1953a) seems to be related to the axonal reflex mechanism. Such mechanism may contribute to vasodilatation also in the dental pulp. The present investigation was designed to study whether electrical stimulation of the inferior alveolar nerve in the cat can influence pulpal circulation and if possible to establish the nature of the dilatory fibres involved.

## METHODS

The experiments were carried out on cats (2-4 kg and 1-2 years old), anesthetized with chloralose (40 mg/kg) and urethane (50 mg/kg). The trachea was cannulated and the blood pressure in the femoral artery was recorded. Body temperature was kept constant at 38°C by heating lamps.

The inferior alveolar nerve was exposed over a length of about 3 cm by removing bone from the mandibular base. The distal stump of the sectioned nerve was stimulated via bipolar silver electrodes at 10-20 Hz, 5 ms and 10 V for periods of ~4 min, using Grass stimulator. A resting period of 10-15 min was allowed between stimulations. In

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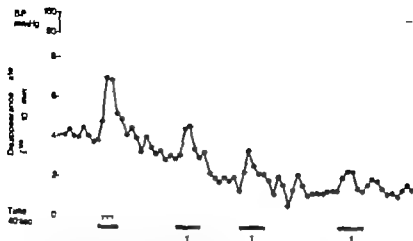


Fig. 1 Influence of superior alveolar nerve stimulation on pupal microcirculation. Cat, young adult. Chloralose-urethane. 1 Stimulation with 10 V, 20 Hz, 5 ms. Each k value represents the running average for 40 period.

third stimulation. Similar results were obtained with atropine (3 mg/kg) and mepyramine (3 mg/kg) together with cimetidine (3 mg/kg) were administered in an analogous way. In all these experiments second response was still pronounced and apparently unaffected by the drugs since there was no significant change in the mean difference between first and second response as compared to control (Table 1). In another series of experiments (5 cats) the nerve was stimulated after pretreatment with all the agents. In this group of animals the first and the second response appeared

unaffected after the drug administration as compared to the control group. Thus in the experimental group the mean response during the first and second stimulations ( $1.41 \pm 0.23$  and  $1.11 \pm 0.39$ ) did not differ significantly from the corresponding responses in the control group ( $1.68 \pm 0.40$  and  $1.01 \pm 0.27$ ).

#### Cutting the inferior alveolar nerve

In 3 cats the nerve remained intact within the mandibular canal until it was cut during the recording of pupal blood flow. In one of these experiments such

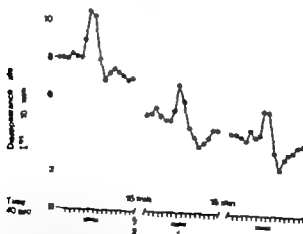


Fig. 2 Influence of inferior alveolar nerve stimulation on pupal microcirculation before and after systemic administration of propranolol. Cat, young adult. Chloralose-urethane. 1 Stimulation with 10 V, 15 Hz, 5 ms. 2, Propranolol 0.5 mg/kg.



Table 1 Increase in  $k$  values reflecting increased pulpal blood flow following inferior alveolar stimulation

All animals were pretreated with phentolamine (3 mg/kg) and the animals in groups A, B and C were given the depot between the first and second stimulations. The results are expressed as mean  $\pm$  S.E. and  $n$  is the number of teeth.

Drug	Stimulation		Mean difference 1-2
	1st	2nd	
Control phentolamine $n=9$	$1.68 \pm 0.40$	$1.01 \pm 0.27$	$0.67 \pm 0.32$
A propranolol $n=4$ (0.5 mg/kg)	$2.42 \pm 0.49$	$1.73 \pm 0.16$	$0.69 \pm 0.13$
B atropine $n=4$ (3 mg/kg)	$1.35 \pm 0.44$	$0.61 \pm 0.03$	$0.74 \pm 0.44$
C mepyramine cimetidine $n=4$ (3 mg/kg)	$1.35 \pm 0.34$	$0.98 \pm 0.02$	$0.37 \pm 0.57$

In some experiments the cervical sympathetic trunk was exposed, cut and stimulated at 3-6 Hz, 1 ms and 6 V for 5-10 min.

A cavity was prepared within the gingival half of the crown in the ipsilateral lower canine tooth. The cavity was deepened by a hand held end cutting burr until the shadow of the pulp was barely visible through a thin layer of dentin. This procedure has previously been reported to cause a minimum of injury to the pulp tissue (Åhlberg & Edwall 1977). Circulatory changes in the pulp were determined by the disappearance technique using a radioactive tracer solution ( $^{125}\text{I}$  80  $\mu\text{Ci}/\mu\text{l}$ ) as iodide dissolved in phosphate buffer with sodium thiosulphate applied as a depot in the tooth cavity. A thin plastic film and Plastibase insulating gel were used to cover the cavity to prevent evaporation. Radioactivity was monitored by an external scintillation detector and counted for 40 s periods. The technical details of this method have previously been described by Bolme & Edwall (1970) and Edwall & Kindlová (1971). Counting was started 5-10 min after the isotope application when the disappearance rate was found to be monoexponential. The disappearance rates were calculated as  $k$  values and changes in this parameter were related to changes in pulpal microcirculation (Edwall 1971; Edwall & Kindlová 1971). Increases in  $k$  values are expressed as the difference between peak values and the mean of 6 prestimulatory values.

Phentolamine (3 mg/kg i.v.) was given before the experiment to prevent vasoconstrictor effects. This dose was previously tested in four animals during stimulation of the cervical sympathetic nerve and was found to abolish the vasoconstrictor response completely after which no increase in disappearance rate occurred. An additional dose of 0.5-1 mg/kg was given every hour to ensure a complete  $\alpha$ -adrenoceptor blockade throughout the experiment.

The changes in  $k$  values observed as a result of inferior alveolar nerve stimulation were compared within the same animal before and after (15-20 min) intravenous administration of propranolol (0.5 mg/kg), atropine (3 mg/kg), mepyramine (3 mg/kg) and cimetidine (3 mg/kg) given separately. Similar comparisons were made between animals which had received all these drugs and untreated controls. Results are expressed as mean  $\pm$  S.E.

## RESULTS

### Inferior alveolar nerve stimulation

Efferent stimulation of the distal stump of the nerve after appropriate  $\alpha$ -adrenoceptor blockade caused a mean increase in the  $k$  values in the ipsilateral tooth of  $1.86 \pm 0.46$  (79 teeth). In experiments with repeated stimulation (9 teeth) the first stimulation produced a greater increase (mean  $1.68 \pm 0.40$ ) than the second (mean  $1.01 \pm 0.27$ ) (Table 1). The responses were further attenuated in experiments where successive stimulations were carried out (Fig. 1) so that the fourth stimulation usually had little or no effect. These results also illustrate the frequent observation that the basal  $k$  values decrease progressively during the course of these experiments. This finding differs from previous results showing that the disappearance rate from dentin usually is monoexponential under resting conditions for at least one hour after the application of the depot (Edwall & Scott 1971; Olgaard & Gællus 1977). A possible explanation for this consistent drop in the  $k$  values is that the exchange function of the capillaries is impaired due to an increased permeability and protein extravasation.

### Attempts to block the vascular response

The nature of the vasodilator mechanism was explored by pharmacological means. Thus, blocking agents were introduced by the systemic route between the first and second stimulations in a series of experiments (16 teeth). The results of one of these experiments in which propranolol (0.5 mg/kg) was given 70 min before nerve stimulation are shown in Fig. 2. As can be seen there was still a pronounced increase in  $k$  values during the

explained by such a vascular reaction. An increase in vascular permeability and protein exudation would impair the capillary exchange of solutes from the tissue and thus reduce the rate of clearance of the tracer from the cavity-tissue interface. Although this late aspect of the vascular reaction has not been further analyzed in this study, it also gives some support for the concept of a reflex mechanism in the dental pulp.

The identity of the vasoactive substance contained in pain fibres is not known. Our findings that vasodilatory responses were attenuated following repeated stimulation may indicate that they are identical to the release of an active neural substance which can be depleted by prolonged electrical stimulation of the nerve. This assumption is strong and may relate to our previous observation that Substance P-like immunoreactivity is contained within fine calibre nerve fibres in the rat dental pulp (Olsson *et al.* 1977) and that small amounts of SPLI can be found in superfusate from the exposed rat pulp following nerve stimulations similar to those used in the present study (Olsson *et al.* 1977b). This release was also noted following repeated stimulations and very little SPLI was found after the second stimulation. We have also demonstrated that synthetic SP has a powerful vasodilatory action in the rat pulp (Olsson *et al.* 1977). Radioimmunoassay of SP in rat pulps revealed amounts of up to 43 ng/g tissue (unpublished). The finding of such high amounts of SPLI is not surprising, since the pulp is richly innervated and recent histological findings in cats indicate that a large proportion of the pulpal nerve fibres belong to the group of unmyelinated fibres (Olsson & Karlsson 1974). The role of these fibres in dental pain has not yet been established but it is likely that vascular reactions in the pulp associated with pain may have a nervous component. In both such fine calibre SP-containing nerve fibres are involved.

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a procedure caused a slight and transient increase in  $k$  values whereas in the other four cats there was no effect.

## DISCUSSION

The present experiments in cats confirm and extend the previous observation in dogs (Tönder & Naess 1978) that electrical stimulation of the mandibular nerve causes increased blood flow in the dental pulp. In addition we have shown that this effect is not mediated by noradrenergic or cholinergic nerves. Our findings that propranolol did not influence the vascular response to inferior alveolar nerve stimulation and that phentolamine failed to reverse the constrictor response to sympathetic stimulation are in agreement with previous observations in dogs and cats showing that there is little evidence that there are  $\beta$ -adrenoceptors in the dental pulp (Edwall 1971; Tönder 1976).

It is not clear if there are cholinergic vasodilator fibres in the dental pulp. Functional studies in dogs (Weiss et al. 1977) and the histochemical demonstration of specific cholinesterase activity related to nerve fibres in the rabbit cat and dog pulp (Avery et al. 1971, 1974; Pohto & Anttila 1972) lend some support to the hypothesis that there is an autonomic vasodilator mechanism in the pulp. The results of the present experiments with atropine show however that acetylcholine is not involved in vasodilatation produced by inferior alveolar nerve stimulation and they do not support the idea of a role for cholinergic vasomotor fibres in the cat dental pulp.

Histamine-like fluorescence has been found in human cat and rabbit pulps (Pohto & Anttila 1972) and mast cell histamine has been suggested to play a part in vasodilatation following electrical stimulation of cutaneous nerves in rats (Kiernan 1975). Since however in the present study the vascular response was not influenced by mepyramine or cimetidine it is unlikely that histamine is involved.

It is thus evident that stimulation of the nerve supply to the dental pulp does not activate common efferent vasodilatory mechanisms and this supports the hypothesis that sensory nerves are involved. Sensory nerves have long been known to mediate vascular reactions in the skin of different species. For example Celander & Folkow (1953a, b) demonstrated in the cat that pain fibres in the skin are involved in vasodilatation induced by local noxious stimulation and by antidromic stimulation of a cut

aneous nerve or dorsal root fibres. It appears that the wellknown axonal reflex mechanism associated with unmyelinated pain fibres is also in the present experiments. The stimulus intensity used in our study is within the known to stimulate afferent C-fibres involved in dilator responses (Hinsey & Garser 1970; Ch. Ladd 1976).

In the present experiments the vasodilator response has been expressed in terms of the disappearance rate ( $k$  value) of indocyanine. According to previous studies changes in disappearance rate are mainly related to changes in capillary blood flow (Edwall 1971). There are however other factors that may influence  $k$  values especially at higher flow rates (Ånggård & Ekelund 1974). For example diffusion barriers between dentin and capillary blood would tend to cause a decrease in  $k$  values. Such a phenomenon could explain the slight decrease in  $k$  values observed during the last part of the stimulation period and more pronounced and transient drop below control values of this parameter after the dilatation (Fig. 2). Furthermore it is possible that during the stimulation a concomitant vasodilatation in neighbouring tissues may reduce the effects on pulpal blood flow due to a drop in pulpal perfusion pressure (Tönder 1976). Accordingly the vasodilator response in pulp may have been underestimated in the present study.

In addition to vasodilatation antidromic stimulation of saphenous and trigeminal nerves in cat has been shown to cause increased vascular permeability (Jancsó, Jancsó-Gabor & Szolcsányi 1967; Chohl & Ladd 1976). This effect usually occurs some minutes after the commencement of the stimulation and has been related to a number of inflammatory mediators. For example prostaglandins have been implicated in neurogenic oedema (Kiernan 1975; Arvier et al. 1977) and a bradykinin-like substance has been found in superfusates from dental pulps of dogs after electrical stimulation of the pulp or the mandibular nerve (Kroeger & Inoki et al. 1973, 1979). It is therefore likely that antidromic vasodilatation in the pulp is also followed by an increase in vascular permeability. This phenomenon may explain the sustained increase in intrapulpal pressure following antidromic stimulation as reported by Kroeger (1968). The progressive decrease in  $k$  values seen in our study after commencement of 1 min stimulation is

# Kallikrein and kinin excretions in dogs during artery constriction and release

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Kallikrein originates intrarenally (Nustad & Lorkowski *et al.* 1976), and the proenzyme is in distal tubular cells (Orstavik *et al.* 1976). It is cleaved into the tubular fluid kallikrein might be potent and active peptides, kinins from kininogen present in plasma. Thus, formation at a distal tubular level is supported by flow analyses (Souch *et al.* 1978).

Urine kallikrein excretion has been used as an index of "intrarenal kinin activity". Reports, however, show that urine kallikrein and kinin excretions might vary independently (Sci *et al.* 1978, Olsen 1978) and it has been suggested that substrate availability rather than enzyme activity might be the rate limiting step in intratubular kinin forming process.

Urine kallikrein is decreased during partial renal artery constriction (Nielsen *et al.* 1976, Kiefer *et al.* 1976) and a transient enhancement of enzyme excretion has been noted following cessation of the constriction (Nielsen *et al.* 1976). Consequently the present study was motivated on the idea that acute changes of renal blood flow might be a useful model to study the interrelationships between kallikrein and urine kinin excretions and shed additional light on the possible physiological significance of the peptides.

Female mongrel dogs (13-23 kg) which had fasted overnight were used for the experiment. The dogs were anesthetized with pentobarbital sodium and surgically prepared with a clamp for maximal constriction placed around the renal artery distal to an electromagnetic flow probe (Olsen 1978). Following completion of surgery the dogs were loaded with 100 ml mannitol in saline (10 ml/min) and 2 ml/min infused throughout the experiment. When urine had stabilized about 45 min after surgery control urine was collected for 15 min. Left renal artery then reduced in two steps. In the first step light reduction (to approximately 90%

of control) ensured maximal autoregulatory vasodilation. In the second step renal blood flow was markedly reduced to approximately 50% of control. At each step the initial 1 ml urine was discarded (in the ureteral caudula dead space volume was 200 µl) and thereafter urine was collected for 15 min. After release of the constriction the experiments were concluded by three 15 min postcontrol urine collections. Urine kallikrein was determined as (1) TABLE-esterase activity (Margolis *et al.* 1974, Olsen *et al.* 1979) and (2) kininogenase activity (Olsen 1978). Subsequent to extraction kinins were assayed biologically (Olsen 1978) and sodium was determined by flame photometry.

The present study shows (Table 1) that the rate of urine kinin excretion decreased by renal artery constriction. The fall of peptide excretion was related to the degree of renal blood flow reduction and was in parallel with decreased urine kallikrein excretion. Upon release of the constriction renal blood flow rapidly normalized. This was followed by a temporary enhancement of kallikrein excretion, whilst urine kinin excretion remained reduced or in fact in 5 of the 10 experiments was temporarily decreased to a level lower than that achieved during the blood flow reductions. This shows that urine kallikrein and urine kinin excretions may vary independently. Apparently enzyme activity is not a major determinant of the intratubular kinin formation. A similar conclusion has been drawn from experiments where urine kinin excretion was increased in association with unchanged or decreased kallikrein as observed in dogs during renal venous constriction (Olsen 1978) or indomethacin treatment to patients with Bartter's syndrome (Vinci *et al.* 1978). Consequently the results suggest that substrate accessibility might be the limiting step in the intratubular kinin forming process. As a corollary this would imply that renal artery constriction might have modified the mechanism(s) by which kininogen becomes available to enzyme. This hypo-

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# te on periodic sweating

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the character of sweat discharge from the sweat glands has been described in several (Randall 1946 Rothman 1954 Boland 1962, 1963, 1964, van Beaumont 1969 Knuyers & 1977). Various periods, ranging from one down to a few sec have been observed.

periods of a few sec are most obvious in periodic sweating and are probably the expression of the autonomic nervous system (Palmer 1951-52, Cotton et al. 1975).

Palmer & Sridharan (1977) found short-period periodic expulsions of sweat by each sweat unit. They claimed that these expulsions are contractions of the myoepithelial cells. The myoepithelial cell origin explaining the periodicity is supported by Nakayama & Takagi (1977) who reported periods of 3-9 s.

Palmer et al (1975) used Fourier analysis to determine individual frequencies in sweating. Similarly Bui et al (1979) showed (by microelectrode recordings from cutaneous nerve fascicles) that motor impulses appear in bursts occurring at intervals, which are integer multiples of 0.6 s. These rhythmically occurring sudomotor responses were accompanied by electrical responses. The results of Bui et al support the theory that the sweat periodicity is mediated by the sympathetic nervous system.

The aim of this work was to study further the periodicity of evaporation prior to and during thermal loading, using a fast responding method for continuous measurement (Nilsson

$D'$  is a constant ( $0.670 \times 10^{-6}$  g/mhPa)

$\frac{\partial p}{\partial x}$  is the vapour pressure gradient (Pa/m)

The evaporation rate is proportional to the vapour pressure difference of two points situated on a line perpendicular to the evaporative surface. The vapour pressure at each point is calculated as the product of the relative humidity and the saturated vapour pressure, the latter a function of the temperature alone. The response time of the instrument, which is limited by the time constants of the temperature and humidity sensors, is short (0.3 s at 25°C). In addition the measurement method influences the evaporation process only to a minimal extent.

The material consisted of 18 healthy subjects (13 men and 5 women, ranging in age from 26 to 37 years). The measurements were performed at an ambient temperature of  $24.8 \pm 2.5^\circ\text{C}$  (mean  $\pm$  S.D.) and a relative humidity of  $34 \pm 10\%$  (mean  $\pm$  S.D.).

The experiment was initiated by letting the subjects sit a few minutes at rest on a cycle ergometer. They then started to exercise on the cycle ergometer (mean work load 65 W) for 10 min, during which time period sweating broke out within a few minutes. During the entire experiment the evaporation rates from the flexor side of the right forearm, were continuously measured by the evaporimeter. The evaporation rates were recorded on an instrumentation tape recorder.

Time intervals of the duration of 1 min were selected. Analysis of the selected passages was performed by autocorrelation (maximum time delay  $\tau = 5$  s) in a mini-computer.

The autocorrelation functions (ACFs) were calculated from the values of the evaporation rates during the exercise period. In all 18 subjects a similar pattern of the ACF was found during the time period prior to active sweating as recorded by the evaporimeter. The average ACF of the 18 subjects is shown in Fig. 1.

A periodicity can be observed in the autocorrelation function. The period is  $0.74 \pm 0.06$  s (mean  $\pm$

measurement principle based on the estimate of the vapour pressure gradient of the air close to the skin. In the vicinity of the evaporative surface the following equation is valid

$$D' \frac{\partial p}{\partial x}$$

where

$D'$  is the amount of water evaporated per unit time and area (g/m<sup>2</sup> h) in this paper expressed as evaporation rate

Table 1 The effect of renal artery constriction (RAC) on kidney parameters

Results are mean  $\pm$  S.E. (V=10)  $-p<0.05$   $-p<0.01$  by Wilcoxon matched pairs signed rank test

	Precontrol 0-15 min	RAC-step 1 15-30 min	RAC-step 2 30-45 min	Postcontrol 45-60 min	60-75 min	75-90 min
Renal blood flow ml/min	151 $\pm$ 2	133 $\pm$ 19*	86 $\pm$ 13	153 $\pm$ 2	161 $\pm$ 1	184
Urine flow ml/min	1.6 $\pm$ 0	1.1 $\pm$ 0.2	0.8 $\pm$ 0.2	1.5 $\pm$ 0.2	1.7 $\pm$ 0.1	1.74
Sodium excr $\mu$ mol/min	118 $\pm$ 26	76 $\pm$ 19*	41 $\pm$ 17	86 $\pm$ 15	11	18
TAME-esterase mEU/min	0.18 $\pm$ 0.03	0.17 $\pm$ 0.03	0.13 $\pm$ 0.04	0.24 $\pm$ 0.03	0.18 $\pm$ 0.03	0.14
Kininogenase U/min	3 $\pm$ 0.4	2.0 $\pm$ 0.4	1.3 $\pm$ 0.3	2.6 $\pm$ 0.5	3.3 $\pm$ 0.4	3.34
Kinin excr ng/min	3.4 $\pm$ 0.7	2.6 $\pm$ 0.5	2.0 $\pm$ 0.4	1.7 $\pm$ 0.7	2.6 $\pm$ 0.8	3.24

thesis however remains to be evaluated. Alternatively though less comprehensible reductions of urine kinin excretion by renal artery constriction might be due to increased intrarenal degradation of kinins. In dogs kininase inhibition by SQ 14725 or SQ 27885 has been shown markedly to enhance the appearance of kinins in urine (Nasjletti et al 1975; Olsen & Arrigoni Martelli 1979) thus suggesting that kininase activity might also determine urine peptide excretion.

The investigations of the role of intrarenally generated kinins are hampered by the lack of specific kinin inhibitors. Infused into the renal artery the peptides are potent vasodilators that produce natriuresis (Jacobson 1970). The present data however do not permit any conclusion about the possible physiological role of kinins formed intrarenally. The immediate normalization of blood flow associated with reduced kinin excretion following cessation of renal artery constriction shows that intratubularly generated kinins exhibited minor (if any) hemodynamic function. Also supporting this view are our previous experiments in dogs which showed that increased intratubular kinin generation by renal venous constriction apparently did not affect renal blood flow (Olsen 1978).

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# Evidence for a histamine $H_2$ receptor involvement in clonidine's antihypertensive effects during multiple dosing

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$\alpha$ -adrenoceptor agonist action has been held to be the main mechanism of clonidine's pressure lowering effect (Schmitt et al. 1971; 1977). That concept has been questioned, involvement of central histamine  $H_2$ -receptors has been suggested by Karppanen et al. (1976). Most of the cited studies have, however, been performed in the anesthetized normotensive animal, injections which allow no conclusions as to antihypertensive mechanism, in particular multiple dosing. During such a circumstance ( $>0.4$  ng/ml) has been found to induce a station dependent blood pressure decrease spontaneous hypertensive rat ((SHR) Christensen et al. 1979) with maximal blood decrease at plasma concentrations of 1-4 ng/ml. At stations above 4 ng/ml the antihypertensive effect is blunted. In the present study the influence of clonidine (a clinically used histamine  $H_2$ -antagonist) is studied on the blood pressure effects of clonidine at low plasma concentrations—giving a pressure response—and at the high concentrations the hypotensive effect is reduced. Rats of the Okamoto strain were used (mean  $200 \pm 10$  g). An indwelling carotid arterial catheter (valvulocid 0.023) and a jugular vein catheter were implanted during barbitone anesthesia and externalized to the back of the neck of the rat. After recovery from the operation (after 1-2 h) the blood pressure was recorded in the conscious freely moving animal by pressure transducer (Cathion Pb 17) connected to Grass polygraph. Clonidine was infused at two consecutive rates according to the technique described by Wagner (1979) and applied by Christensen et al. (1979). The technique enables steady state concentrations of drug to be reached rapidly. Plasma concentrations of clonidine of 1 ng/ml and 10 ng/ml were used. At the end of the experiment which lasted up to 3 h, blood samples for clonidine determination were taken to ensure that the predicted

concentration was obtained. Clonidine was assayed by a gas liquid chromatography method (Edlund & Paulow 1977) as described (Christensen et al. 1979). Clonidine (kindly supplied by Smith Klein & French) 500-1000  $\mu$ g was given as a bolus i.v. 60 min before steady state concentrations of clonidine and during steady state conditions. 5-9 experiments were performed at each steady state level. Values mean  $\pm$  S.D. are given as % decrease of blood pressure in relation to pretreatment period (mean value of 3 measurements in each rat during 1 h). Significance between observations were obtained by the paired *t* test.

The mean values of the blood pressure in the freely moving rat was  $175 \pm 10$  mmHg. Clonidine at a plasma concentration of 1 ng/ml reduced the blood pressure  $7 \pm 3.4\%$  at 60 min and  $15 \pm 3.4\%$  at 180 min after the start of the infusion (Fig. 1). No blood pressure decrease was found at 60 min at plasma concentrations of 10 ng/ml but after 180 min a slight decrease of  $8 \pm 4.3\%$  was observed. Clonidine alone (500-1000  $\mu$ g) did not influence the blood pressure or the heart rate. Pretreatment with cimetidine abolished the hypotensive effect of clonidine (1 ng/ml) (see Figs. 1 and 2) e.g. the blood pressure was unchanged or somewhat elevated ( $6 \pm 3\%$ ) compared to the pretreatment levels. Cimetidine did not influence the blood pressure effect when the plasma concentration was 10 ng/ml. Bolus injection of cimetidine did not affect clonidine's antihypertensive effect when hypotension was achieved. The plasma concentrations of clonidine (1 and 10 ng/ml) were not altered by cimetidine—the measured values at the end of the infusion was  $0.91 \pm 0.2$  and  $11 \pm 2.3$  ng/ml—indicating that the kinetics of clonidine were not changed by cimetidine.

It has been postulated that central histaminergic neurons participate in central regulation of blood pressure (Schwartz 1979; Finch & Hicks 1976). Metiamine, a histamine  $H_2$ -antagonist by itself has not



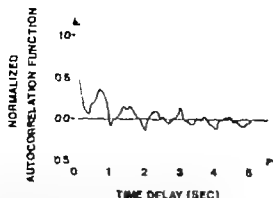


Fig. 1 The calculated autocorrelation function (mean of 18 subjects) of the evaporation rate during exercise on a cycle ergometer.

S.D.) A periodical pattern was also found although less pronounced after the onset of sweating. The difference between our result and many of the earlier reported periods of the evaporation rate variations probably has its origin in the different measuring techniques used and in different experimental conditions. However the result reported in this paper does not exclude the existence of other periods reported by earlier investigators. Our result (a sweating period of 0.74 s) is in agreement with the periodicity of around 0.6 s of the sudomotor nerve impulses reported by Bini et al (1979). These similarities suggest that the variations in the evaporation rate directly reflect the periodic activity in these nerves.

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# Evidence for a histamine H<sub>2</sub>-receptor involvement in clonidine's antihypertensive effects during multiple dosing

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$\alpha$ -adrenoceptor agonist action has been held as the main mechanism of clonidine's pressure lowering effect (Schwartz et al. 1971, 1977). That concept has been questioned by involvement of central histamine H<sub>2</sub>-receptors suggested by Karppanen et al. (1976). Most of the cited studies have, however, been done in the anesthetized normotensive animal with injections which allow no conclusions as to antihypertensive mechanisms in particular during multiple dosing. During such a circumstance ( $>0.5$  ng/ml) has been found to induce a tration dependent blood pressure decrease in spontaneously hypertensive rat (SHR) (Christensson et al. 1979) with maximal blood decrease at plasma concentrations of 1-4 ng/ml. At doses above 4 ng/ml, the antihypertensive effects. In the present study the influence of clonidine in clinically used histamine H<sub>2</sub>-antagonists studied on the blood pressure effects of clonidine at low plasma concentrations—giving a dose response—and at the high concentrations the hypotensive effect is reduced.

of the Okamoto strain were used (mean 100-110 g). An indwelling carotid arterial catheter (Intracath 0.025) and a jugular vein catheter were implanted during barbitone anesthesia and exteriorized to the back of the neck of the rat. After recovery from the operation (after 1-2 days) the blood pressure was recorded in the conscious freely moving animal by a pressure transducer (Statham Po 32) connected to Grass polygraph. Clonidine was infused at 10 consecutive rates according to the technique described by Wagner (1971) and applied by Christensson et al. (1979). This technique enables steady state concentrations of the drug to be reached rapidly. Plasma concentrations of clonidine of 1 ng/ml and 10 ng/ml were reached. At the end of the experiment which lasted up to 3 h blood samples for clonidine determination were taken to ensure that the predicted

concentration was obtained. Clonidine was assayed by a gas liquid chromatography method (Edlund & Paulow 1977) as described (Christensson et al. 1979). Cimetidine (kindly supplied by Smith Klein & French) 500-1000  $\mu$ g was given as a bolus i.v. 60 min before steady state concentrations of clonidine and during steady state conditions. 5-9 expts. were performed at each steady state level. Values mean  $\pm$  S.D. are given as % decrease of blood pressure in relation to pretreatment period (mean value of 3 measurements in each rat during 1 h). Significance between observations were obtained by the paired *t*-test.

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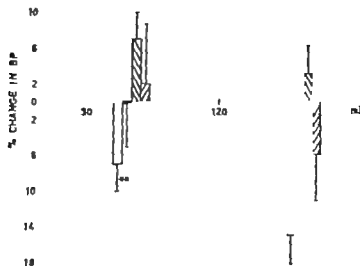


Fig 1 Hypotensive effect of clonidine alone at steady state plasma concentration ( $C_{pss}$ ) of 1 ng/ml (open bars) and 10 ng/ml (black bars) and cimetidine pretreatment 500  $\mu$ g i.v. at corresponding steady state concentrations of 1 ng/ml (□) and 10 ng/ml (■). Values are mean  $\pm$  S.D. ( $n=5-9$ ). \* denotes significance at the 1% level when compared to the effect after pretreatment with cimetidine.

been found to influence blood pressure however (Finch & Hicks 1976). Although cimetidine initially was claimed to be devoid of central nervous effects, a recent report has shown that during multiple

dosing cimetidine causes mental confusion in patients which indicates that the drug passes the blood-brain barrier (Schentag et al 1979). In the present study cimetidine attenuated the antihypertensive effect of clonidine. This finding is in accordance with a previous study which showed that clonidine blood pressure reduction in the anaesthetized rat was reduced by methylnadate (Karppanen et al 1976). Histamine ( $H_2$ )-receptors might be involved in the antihypertensive mechanism of clonidine, and the results are supportive of the concept (Finch & Hicks 1976) that histamine receptors are involved in the regulation of blood pressure.

The skilful technical assistance of Mrs Lagergren is greatly acknowledged. The investigation was supported by the Swedish medical research council B-79-0474-01.

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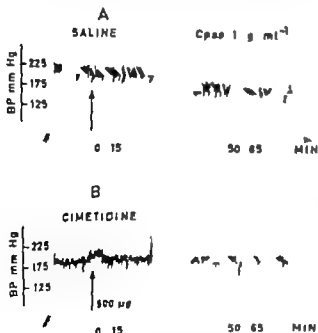


Fig 2 Influence of saline (A) and cimetidine (500  $\mu$ g) (B) on clonidine's hypotensive effect at steady state concentrations of 1 ng/ml. The tracings are from 2 separate rats. The pretreatment injection was given 60 min before steady state concentrations were achieved. Horizontal axis: time course. Vertical axis: mean arterial pressure (mmHg).

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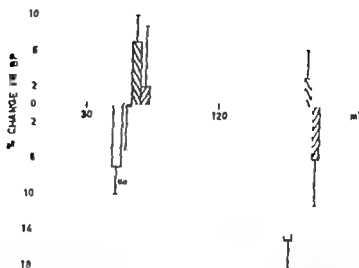


Fig. 1 Hypotensive effect of clonidine alone at steady state plasma concentrations (C<sub>pre</sub>) of 1 ng/ml (open bars) and 10 ng/ml (black bars) and cimetidine pretreatment 400 µg i.v. at corresponding steady state concentrations of 1 ng/ml (□) and 10 ng/ml (■). Values are mean ± S.D. (n=5-9). \* denotes significance at the 1% level when compared to the effect after pretreatment with cimetidine.

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The skilful technical assistance of Mrs Lager Löf is greatly acknowledged. The investigation was supported by the Swedish medical research council B-79/017-01.

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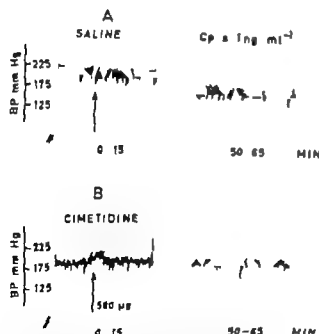


Fig. 2 Influence of saline (A) and cimetidine (400 µg) (B) on clonidine's hypotensive effect at steady state concentrations of 1 ng/ml. The tracings are from separate rats. The pretreatment injection was given 60 min before steady state concentrations were achieved. Horizontal axis: time course. Vertical axis: mean arterial pressure mmHg.

# The mode of action of botulinum toxin

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Botulinum toxin (BoTx) blocks irreversibly and with high specificity the quantal release of acetylcholine from motor nerve terminals. The extreme potency of the toxin, only a few hundred molecules required to block each synapse (Härlig & Larsson 1979) makes it interesting to speculate on its mode of action. Taking in account the evidence that transmitter release facilitates the release of BoTx it has been suggested that the toxin molecule might block the channel or the release site from which acetylcholine quanta are released and that the toxin can only enter into the release position when there is opening of either a strong closed site or a site created de novo by nerve stimulation (Larsson 1976).

The purpose of the present study was to test this hypothesis. 4-aminopyridine is a drug which, by increasing the influx of calcium ions into nerve terminals in response to nerve stimuli, causes a large quantal release (several thousands of quanta) of transmitter (Molgo et al 1977; Lundh 1978). As shown by Heuser et al (1979) the number of synaptic vesicle openings in the nerve terminal revealed by freeze-fracture technique is similarly raised by the drug. Once BoTx has produced a molecular block, 4-AP is a very effective antidote (Lundh et al 1977).

It is concluded that the previously mentioned hypothesis regarding the mode of action of BoTx was correct, one could expect that if BoTx and 4-AP administered together the toxin should be able to block not only the normally activated release for acetylcholine but also the new sites opened in the presence of 4-AP. Hence one could expect that with a combined administration of BoTx and 4-AP the number of release sites blocked should be more than with BoTx alone and as a consequence more 4-AP once the block was established, should be more effective or even ineffective in restoring neuromuscular transmission.

Experiments were carried out on the extensor digitorum longus muscle of male Sprague Dawley rats.

The muscle was poisoned by a single subcutaneous injection of BoTx type A in the hind-leg as described by Cull-Candy et al. (1976). The dose of the toxin caused complete paralysis of the leg lasting several weeks. Some of the animals received at the time of poisoning an intraperitoneal injection of 4-AP (3 mg/kg b.wt.) or 1 h after BoTx poisoning 2.5 mg/kg 4-AP and 4 h later an additional 1.25 mg/kg of the drug. These doses of 4-AP were the maximal tolerated by the animals, larger doses causing generalized convulsions and death 4 days after BoTx poisoning. The extensor digitorum longus muscle with its motor nerve was removed under ether anaesthesia from animals given only BoTx and from animals given BoTx+4-AP placed in a constant temperature bath (30°C) and perfused with

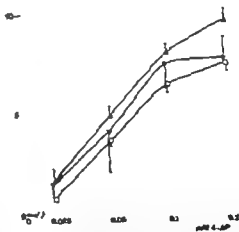


Fig. 1 Isometric twitch tension of isolated extensor digitorum longus muscles in response to indirect stimulation at 0.1 Hz. Dose-response curves are given for cumulative increasing concentrations of 4-AP on 9 muscles treated with BoTx only (O), 3 muscles treated with BoTx and one 4-AP injection (Δ) and 5 muscles given BoTx and 2 4-AP injections (◻). There is significant difference ( $p < 0.05$ , Student's *t*-test) between the upper and lower curve at the two highest drug concentrations. Each value is the mean  $\pm$  S.E.



# Carbonic anhydrase in the lung

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Carbonic anhydrase activity has been demonstrated in lung tissue of human fetus (Berferstam 1952), rhesus monkey (Fisher 1961), fetal lamb (Johnson & Waxman 1976), adult rabbit (Effros et al. 1974) and rat (Maren 1967; Crandall & O'Brady 1976). The localization and function of the enzyme in the lung of these and other mammalian species remains, however, obscure. The aim of the present study was to clarify the distribution of carbonic anhydrase in rat and monkey lung by a histochemical technique.

Experiments on adult male Sprague-Dawley rats and cynomolgus monkeys were performed *in vivo* with saline followed by 2.5% glutaraldehyde buffered to pH 7.4. Small pieces of tissue were then immersed in the fixative for 2 h, embedded in JB-4 plastic, sectioned and stained for electron microscopy. Histochemical method for the histochemical demonstration of carbonic anhydrase activity (Maren 1967) is used in a slightly modified form and by Ruddenström (1976). The method and the results have been discussed by Lönnérholm (1979).

Controls were run with 10  $\mu$ M acetazolamide added to the incubation medium, and this concentration of the inhibitor always abolished visible staining.

In the rat lung staining was found only at the structures which separate capillaries and alveoli, i.e. the alveolar-capillary barrier (Fig. 1). In the electron microscope the precipitate was found mainly at the capillary side (Fig. 1, arrows) of this barrier which consists of capillary endothelium, basement membranes and alveolar epithelium (Weibel 1969). The exact localization of the staining is not clear at present, however. Those parts of the capillary walls which were not in close contact with alveoli were unstained (Figs. 1, 2). Where alveoli were not in close contact with capillaries, their lining structures were unstained (Fig. 1, arrows).

In the monkey lung another staining pattern was found. Stain deposits were seen along the whole circumference of the capillaries (Fig. 3). The structures lining the alveoli were unstained at least

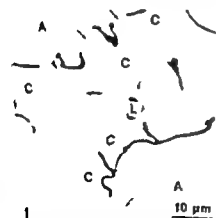


Fig. 1. Rat lung. Stain deposits are confined to the structures separating capillaries, C, and alveoli, A. Note how alveoli are not in close contact with capillaries; these structures are unstained (arrows). One capillary was in unusual location; it is labeled L. Incubation time 5 h. Light microscopy. Phase contrast.



Fig. 2. Rat lung. The stain is located mainly at the capillary side (arrow) of the structures separating capillary C and alveoli A. The capillary wall on the contrary side, which is in contact with interstitial tissue I, is unstained. Incubation time 6 h. Electron microscopy.



oxygenated medium of the following composition in mM: NaCl 135, NaHCO<sub>3</sub> 15.0, Na<sub>2</sub>HPO<sub>4</sub> 1.0, KCl 5.0, CaCl<sub>2</sub> 2.0, MgCl<sub>2</sub> 1.0, glucose 11.0. The pH of the solution was 7.2–7.3.

Isometric twitch tensions were recorded by a Grass FT-03 transducer connected to an ink writing oscillograph. The resting tension of the muscle was adjusted to give maximal twitch response. The nerve was stimulated by a glass capillary suction electrode with supramaximal voltage and pulses of 0.05 ms duration at 0.1 Hz. Cumulative dose-response curves for the effectiveness of 4-AP to restore neuromuscular transmission were constructed allowing 10 min between each increase in drug concentration.

As shown by Fig. 1, 4-AP was somewhat more effective in restoring neuromuscular transmission in those preparations which 4 days previously had received both BoTx and 4-AP as compared to BoTx only. Thus there is no indication that BoTx preferentially blocks activated acetylcholine release sites in the nerve terminal membrane. It appears necessary to consider some other mechanism of action of the toxin on the probability of transmitter release, possibly one reflecting the calcium requirement of the release process as suggested by Cull-Candy et al. (1976).

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# Carbonic anhydrase in the lung

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Carbonic anhydrase activity has been demonstrated in tissue of human fetus (Bergerstam 1952), rhesus monkey (Fisher 1961), fetal lamb (Fisher & Waxman 1976), adult rabbit (Effros et al 1978) and rat (Maren 1967, Crandall & O'Brady 1978). The localization and function of the enzyme in the lung of these and other mammalian species remains, however, unknown. The aim of the present study was to clarify the distribution of carbonic anhydrase in rat and monkey lung by a histochemical technique.

Groups of adult male Sprague-Dawley rats and cynomolgus monkeys were perfused *in situ* with saline followed by 2.5% glutaraldehyde buffered to pH 7.4. Small pieces of tissue were then immersed in the fixative for 2 h, embedded in JB-4 plastic, sectioned and stained for electron microscopy. Histochemical method for the histochemical demonstration of carbonic anhydrase activity (Maren 1967) was used as slightly modified form described by Riddervik (1976). The method and the results have been described by Lönnérholm (1979).

Controls were run with 10  $\mu$ M acetazolamide added to the incubation medium and this concentration of the inhibitor always abolished visible staining.

In the *rat lung* staining was found only at the structures which separate capillaries and alveoli, i.e. the alveolar-capillary barrier (Fig. 1). In the electron microscope the precipitate was found mainly at the capillary side (Fig. 2 arrows) of this barrier which consists of capillary endothelium, basement membranes, and alveolar epithelium (Weibel 1969). The exact localization of the staining is not clear at present, however. Those parts of the capillary walls which were not in close contact with alveoli were unstained (Figs 1, 2). Where alveoli were not in close contact with capillaries, their lining structures were unstained (Fig. 1 arrows).

In the *monkey lung* another staining pattern was found. Stain deposits were seen along the whole circumference of the capillaries (Fig. 3). The structures lining the alveoli were unstained, at least



Fig. 1. Rat lung. Stain deposits are confined to the thin barrier separating capillaries, C, and alveoli, A. Note how alveoli are not in close contact with capillaries, and lung structures are unstained (arrows). One capillary with an unstained leukocyte, L. Incubation time 5 min. Light microscopy. Phase contrast.



Fig. 2. Rat lung. The stain is localized mainly at the capillary side (arrows) of the structures separating capillary C and alveolus A. The capillary wall on the contrary side which is in contact with interstitial tissue, I, is unstained. Incubation time 6 min. Electron microscopy.

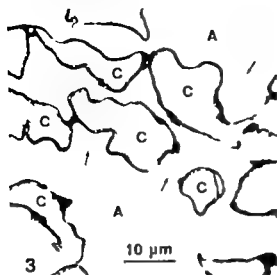


Fig 3 Monkey lung. Stain deposits are present along the whole circumference of the capillaries C. The lining of the alveoli A is unstained at least where they are not in close contact with capillaries (arrows). Incubation time 8 min. Light microscopy. Phase contrast.

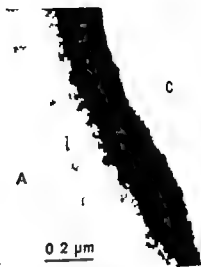


Fig 4 Monkey lung. High power magnification of structures separating a capillary C and alveolus A, showing stained capillary endothelium. Incubation time 8 min. Electron microscopy.

where they were not in close contact with capillaries (Fig. 3 arrows). In the electron microscope the staining was found at the capillary side of the alveolar-capillary barrier (Fig. 4). Although the results suggest that carbonic anhydrase is located in the capillary endothelium, further work is needed to clarify the ultrastructural localization of the enzyme.

A major difficulty in the study of carbonic anhydrase in various tissues is to avoid contamination with enzyme from erythrocytes. In the present study the following points argue against the near-at-hand suspicion that the observed staining could be due to a fixation artifact and caused by enzyme from ruptured erythrocytes: 1) A similar staining pattern was seen in unperfused lungs and extensively perfused lungs where no or very few erythrocytes remained. 2) The whole circumference of the rat lung capillaries did not stain, which one should expect if the staining was due to enzyme from erythrocytes.

The function of carbonic anhydrase in the lung is not clear at present. It has been suggested that the enzyme facilitates  $\text{CO}_2$  transport through the lung tissue (Enns & Hill 1975). The present demonstration that the enzyme is indeed located at the alveolar-capillary barrier in the rat and monkey lung could agree with a role for it in the exchange of  $\text{CO}_2$  over this barrier.

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# ulatory effects of decerebration e unanesthetized spontaneously ntensive rat<sup>1</sup>

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hypertension developed in the spontaneously  
tensive rat (SHR) is dependent on the activity  
sympathetic nervous system (see Yamori  
Thus the brain is of fundamental importance  
maintenance of the sympathetic activity is well

This is true also for the SHR as indicated  
finding that pithing or ganglionic blockade  
sets the blood pressure to the same low level  
SHR as in the normotensive control rat  
1969 Shibayama et al 1971 Tranchesi  
Albrecht et al 1975) Some studies indicate  
that brain mechanisms could be actively in-  
volved (responsible for) the development of  
armon in the SHR (Haessler et al 1972  
et al 1976 Nakamura & Nakamura 1977)

blood pressure and heart frequency of  
ed Dawley normotensive unanesthetized rats  
ned markedly after decerebration or a pre-  
balamic transection (Trolin 1975) This would  
to indicate that in the rat brain centers rostral  
lesions normally have a net inhibitory effect  
e sympathetic outflow. In view of the pro-  
difference in the central nervous activity in  
HR compared to the normotensive rat, it was  
of interest to study the circulatory effects  
decerebration also in the unanesthetized SHR.

**Methods** Male SHR of the Okamoto strain, Wistar  
normotensive rats (WK) (Møllegaard, Denmark),  
Sprague Dawley (SD) (Arborelius, Sweden) rats  
250 g) and two types of brain lesions were  
made. For decerebration at the midcollicular level  
the rat was placed immediately caudal to the supra-  
scapular and the transection rostral to the hypo-  
thalamic (prechiasmatic transection) the skull was  
drilled on both sides of the supra scapular just rostral  
to the supra scapular. After opening of the skull the  
rat was returned and the rat was left to recover from  
the operation for 7 days. On day 3 catheter was in-  
serted into the left common carotid artery in a described  
manner (1974). On day 4 the basal blood pressure and

heart frequency were recorded for at least 70 min. There  
after the rat was disconnected from the transducer and  
under a short-acting halothane (Halothane<sup>TM</sup> Hoechst)  
anesthesia the old operation wound of the skull was  
opened and the decerebration or prechiasmatic transec-  
tion was performed blindly with a spatula through the pre-  
made opening(s) of the skull (see Trolin 1975). The rat  
was quickly re-connected to the polygraph and the blood  
pressure and heart frequency were recorded for one hour.  
The duration of the anesthesia was never more than 3  
min. A few non-lesioned rats recovered within a few  
minutes from this anesthesia. At the end of each experi-  
ment the level and completeness of the transection was  
checked. Only completely and correctly transected rats  
were considered for statistical analysis. Student's *t*-test  
after analysis of variance by two independent criteria of  
classification or Student's *t*-test alone was performed to  
calculate significance of differences. *P*-values less than  
5% were regarded as significant.

**Results** The basal blood pressure before decere-  
bration was significantly higher in the SHR ( $154 \pm 6.3$   
mmHg  $\pm 6.3$ ) than in the WK or SD rats ( $124 \pm 7.3$   
and  $120 \pm 6.4$  respectively). The basal heart fre-  
quency was equal in the SHR and SD rat ( $385 \pm 15$   
and  $372 \pm 12$  resp.) but significantly lower  
in the WK rat ( $300 \pm 3.3$ ).

After decerebration the blood pressure and heart  
frequency increased significantly in the SD and WK  
rats. The increase in the SD group showed less  
variation than in the WK group. However in the  
SHR the decerebration neither produced increase  
in blood pressure nor in heart frequency.

One hour after decerebration the blood pres-  
sures of the 3 groups were not significantly different  
from each other while the heart frequencies of the  
SD and WK groups were both significantly higher  
than the heart frequency of the SHR group (Fig. 1)

A preliminary report was presented at the congress of  
SB P.C. 1978, São Paulo, Brazil.

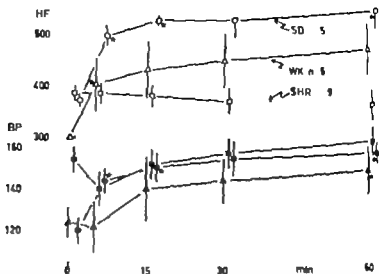


Fig. 1. Changes in mean arterial blood pressure (BP, mmHg, closed symbols) and heart frequency (HF, beats/min, open symbols) after decerebration (time 0 min) in Sprague Dawley (SD, circles,  $n=5$ ), Wistar Kyoto (WK, triangles,  $n=5$ ) and spontaneously hypertensive (SHR, squares,  $n=9$ ) rats. The rats were anesthetized for ~3 min during the decerebration. The values are means indicated with S.E. Values significantly different ( $P < 0.05$ ) from the basal value of the same group are indicated with stars.

Essentially the same results were obtained after a prehypothalamic transection of the brain (data not shown).

**Discussion** The pronounced increases in blood pressure and heart frequency after decerebration of unanesthetized SD rats were similar to the effects reported earlier (Trolin 1975). The same results were now also obtained with WK rats. The only difference was that in this group the development of hypertension and tachycardia showed more variations than in the SD group. On the other hand in the SHR decerebration did not produce any circulatory effects. That the pressure did not increase after decerebration in this group could of course be explained by the high initial pressure. However neither did the heart frequency increase after decerebration in this group. The easiest explanation for this difference between SHR, WK and SD rats seems to be that decerebration causes an increase in sympathetic activity in the WK and SD groups but not in the group of SHR. It seems safe to conclude that the mechanism responsible for this difference between the strains is to be found within the CNS. One possible explanation could be that an inhibitory pathway descending from higher centers (e.g. Folkow et al. 1959) is normally active in normotensive rats but of less importance, maybe even lacking, in the SHR.

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# nal EDL and diaphragm muscles differ eir sensitivity to tetrodotoxin

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nal skeletal muscle the action potential is  
by blocked by tetrodotoxin (TTX  $10^{-6}$  M)  
966). However, after denervation resistance  
appears (Redfern & Theisfeldt 1971) along  
other striking membrane changes (for a review  
rves 1976). In a recent work we showed that  
denervation of the rat extensor digitorum  
(EDL) muscle induces TTX resistance not  
in the denervated but also, although to a lesser  
in the endplate region of adjacent active  
ated fibres (Cangiano & Lutzemberger  
This and other (Jones & Vrbová 1974; Lomo  
rsgaard 1976; Brown et al. 1978) findings  
at that nerve degeneration induces denerva-  
like changes in muscle also through mechan-  
not depending on muscle inactivity. However,  
results have been questioned by Tiedt et al.  
a) to report the presence of TTX resistance  
in endplate region of many normal EDL fibres  
b) although in an earlier report (Tiedt et al.  
c) such fibres are said to be rare ( $<1\%$ ). They  
emphasize the presence of TTX resistance in  
endplate region of many normal diaphragm  
as reported by Theisfeldt et al. (1974).

in order to be able to interpret the effects of  
of denervation correctly it is important to  
study whether the remaining innervated fibres  
develop a genuine increase in TTX resistance. We  
therefore reexamined the TTX resistance of  
fully innervated EDL and diaphragm muscles.  
Normal diaphragm and EDL muscles from male  
Sprague-Dawley rats (280-340 g) were dissected free and  
placed in a chamber containing 25 ml of an oxy-  
genated mammalian Ringer's solution at  $28^{\circ}\text{C}$ , pH  
7.3 containing TTX  $10^{-6}$  M. In the endplate  
region, identified by the presence of fast rising mini-  
ature endplate potentials, the fibres were penetra-  
ted by closely adjacent conventional recording  
electrodes and current injecting micropipettes. The resting  
membrane potential (RMP) was set at  $-100$  and

$-120$  mV by a continuous hyperpolarizing current  
and action potentials were generated by superim-  
posed depolarizing current pulses of 12 ms duration.  
The rate of rise of the action potential was meas-  
ured simultaneously and its maximal value taken as  
a measure of the resistance to TTX. This value  
was influenced by the intensity of the depolarizing  
pulse: strong pulses eliciting action potentials with  
shorter latencies and faster rates of rise (see an  
example in Fig. 1B). The pulse was therefore ad-  
justed to generate action potentials at constant  
latencies of 4 and 2 ms. In this way the EDL and  
the diaphragm muscles could be examined under  
strictly comparable conditions in the same bath.

As shown in Fig. 1A and Table 1 most of the  
fibres in the normal diaphragm give action poten-  
tials at 4 ms in the presence of  $10^{-6}$  M TTX although  
the rates of rise (and amplitudes) are much reduced  
compared to normal. This is in agreement with  
Theisfeldt et al. (1974). In contrast, most of the fibres  
in the EDL fail to give any regenerative response  
at 4 ms in agreement with our earlier report (Can-  
giano & Lutzemberger 1977). At a latency of 2 ms

Table 1. Maximal rate of rise (volt/sec mean  $\pm$  standard error) of regenerative response elicited in TTX  $10^{-6}$  M from the end-plate region of normal diaphragm and EDL muscle fibres at two different delays (2 and 4 ms) and resting potentials set at  $-100$  and  $-120$  mV

Each group is composed of 32 to 38 fibres from 4 animals

	2 ms	4 ms
EDL		
100 mV	$2.5 \pm 1.5$	0.0
120 mV	$39.1 \pm 5.3$	$5.4 \pm 1.9$
Diaphragm		
100 mV	$51.9 \pm 4.4$	$27.9 \pm 3.0$
120 mV	$75.9 \pm 6.2$	$56.4 \pm 4.3$

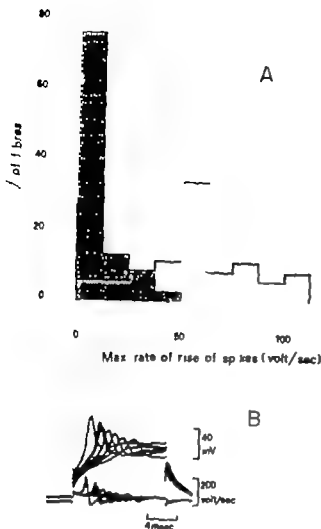


Fig. 1 (A) Distribution of resistance to TTX measured as the maximal rate of rise of the action potential in normal diaphragm fibres (open columns, 36 fibres, 4 muscles) and in normal EDL fibres (dashed columns, 38 fibres, 4 muscles). All measurements performed at the neuromuscular junction, resting membrane potential set at  $-120$  mV by DC hyperpolarizing current, constant delay of 4 ms between onset of the depolarizing pulse and firing of the regenerative response. (B) Regenerative responses of a diaphragm fibre in TTX illustrating the dependence of the rate of rise on the delay between onset of the depolarizing pulse and attainment of threshold. The first derivative (bottom trace) of membrane potential changes (top trace) was obtained with an RC circuit (100 kohms, 100 pF). Resting potential set at  $-100$  mV.

and a membrane potential of  $-170$  mV, however small action potentials appear whose rates of rise are much slower than those elicited in the diaphragm under the same conditions (Table 1).

It is clear from these results that the normal diaphragm is considerably more resistant to TTX in the endplate region than the EDL. The reason for this is unclear. It is unlikely to be due to the

presence of a larger number of slow fibres in the diaphragm (Korneliusson & Wærhaug 1977) because the slow soleus is affected as strongly as the fast EDL (unpublished observations).

The most important conclusion of this work is that under the conditions used in our earlier work (Cangiano & Lutzemberger 1977) where action potentials were elicited at a constant interval of 4 ms, there is essentially no resistance to TTX in the endplate region of normal EDL fibres. For the development of TTX resistance in the partially denervated muscles previously reported by (Cangiano & Lutzemberger 1977) is a real phenomenon indicating that nerve degeneration correlates with inactivity of the muscle (Lomo & Westgaard 1976) to its response to denervation. A different conclusion reached by Tjedi et al. (1976) could arise because they used different and inconsistent criteria for assessing the TTX-resistance.

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# Electrical field stimulation of myometrial strips from non-pregnant and pregnant guinea pigs

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ELMÉR, P., ALM, P. & THORBERT, G. Electrical field stimulation of myometrial strips from non-pregnant and pregnant guinea-pigs. *Acta Physiol Scand* 1980, 108: 209-213. Received 28.5.1979. ISSN 0001-6772. Departments of Physiology, Histology, Pathology and Obstetrics and Gynecology, University of Lund, Sweden.

Myometrial tissue strips from term pregnant and non-pregnant guinea-pigs were studied *in vitro* by electrical field stimulation, and the adrenoceptor sensitivity to exogenously applied noradrenaline was determined. The induced response was characterized by various pharmacological and nerve blocking drugs. In previously contracted strips from non-pregnant animals field stimulation provoked a relaxation probably mediated via postganglionic adrenergic nerve terminals and myometrial  $\beta$ -receptors. In strips from myometrial tissue surrounding fetuses, no response was seen after field stimulation. Relaxation induced by exogenous noradrenaline was more pronounced and provoked at lower concentrations in pregnant than in non-pregnant tissue. However, in non-fetus-carrying uterine horns in mid-pregnant pregnancies field stimulation still induced relaxation, and the sensitivity to exogenous noradrenaline was less than in the contra-lateral fetus-carrying horn. The physiological findings were compared with the endogenous level of uterine noradrenaline transporter and the presence of adrenergic nerves, demonstrated by the Falck-Hallarp fluorescence technique. It is suggested that the altered effects at term pregnancy are due to pregnancy induced postganglionic denervation of the adrenergic terminals in the fetus-carrying uterine horn. This is accompanied by an increased postsynaptic adrenoceptor sensitivity to the transmitter as analogous with denervation supersensitivity after adrenergic denervation. Furthermore the present results verify the recent structural and biochemical findings of an adrenergic nerve degeneration in fetus-carrying myometrial tissue during pregnancy probably representing a very special neuro-endocrine neuron-target relationship.

**Key words:** Myometrium, field stimulation, receptor sensitivity, denervation.

Nerves from many species including man consist of adrenergic nerves located both in blood vessels and in association with smooth muscle cells (see review by Marshall 1970). The adrenergic nerves are derived from both short and long adrenergic neurons with their cell bodies located pre- or paravertebrally and from adrenergic neurons located very close to the effector tissue (Owman, Sjöberg & Sjöstrand 1974). During pregnancy the level of uterine noradrenaline transporter is highly reduced, probably due to structural degeneration of the adrenergic nerve plexus (Owman et al. 1975; Thorbert 1979). At the time of parturition this is related to the absence of tyrosine hydroxylase ac-

tivity, a rate-limiting noradrenaline synthesizing enzyme (Alm et al. 1979) and the absence of the adrenergic axonal transmitter uptake mechanism (Alm et al. 1979b). Further during advancing pregnancy ultrastructural signs appear characteristic of adrenergic nerve degeneration (Sporrøng et al. 1978).

By the stimulation of extrinsic nerves to the uterus motor responses can be elicited. These are the result of preganglionic nerve stimulation due to the occurrence of parasympathetic ganglia and short adrenergic neurons located very close to the uterus (see review by Marshall 1970). On the other hand by electrical field stimulation of myometrial tissue strips in combination with pharmacological



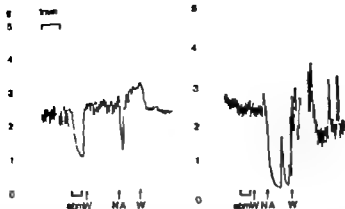


Fig. 1 Responses of spontaneously contracted myometrial strips from guinea-pig with unilateral pregnancy to electrical field stimulation (stimW) and noradrenaline (NA). Left: strip from the empty uterine horn, right: strip from the contralateral fetus-carrying horn excised in the same bath. At W the bathing solution was renewed 3 times.

taken from fetus-carrying horns in 7 out of 9 sex guinea-pigs and in strips from 4 animals treated with 6-OH-DA there was no response to test stimulation.

Effect of noradrenaline caused relaxation of the pregnant (fetus-carrying) and the non-pregnant myometrium, the threshold concentrations lower and the responses greater in the pregnant myometrium (Table 1). In strips from 2 non-pregnant animals the initial relaxation was followed by small contraction which was abolished after addition of dihydroergotamine, 6 µg/ml.

Responses to electrical stimulation and to noradrenaline administration of myometrial strips from animals with unilateral pregnancy are shown in Fig. 1. Electrical field stimulation did not produce any effect in the fetus-carrying strips whereas in the empty non-fetus-carrying strips a relaxant effect was seen. Further the addition of noradrenaline produced relaxation in myometrial strips of both types of horns. However the effects were more marked in the strips from fetus-carrying horns than in the strips from empty horns.

## DISCUSSION

In the present study electrical field stimulation of isolated myometrial strips from estradiol-17β-treated animals produced a relaxant effect.

Short stimulation pulses were used in order to avoid direct stimulation of the muscle cells (cf. Swedin 1971). The response was completely abolished after the administration of tetrodotoxin, suggesting that it was nerve-mediated. The total abolishment of the field stimulation-induced relaxation after the administration of guanethidine being a potent adrenergic neuron blocking drug (see Costa 1966, Abba & Dodd 1974) or in animals pre-treated with 6-OH-DA well-known for its ability to selectively degenerate adrenergic nerves (Thoenen & Tranzer 1968) strongly suggests that in the field stimulation experiments the nerves mediating the response are adrenergic. This is in line with previous histochemical findings, demonstrating adrenergic but no certain cholinergic nerve fibres in the guinea pig

Table 1 Threshold concentrations µg/ml and inhibitory responses, g to 20 µg/ml of noradrenaline in the guinea-pig uterus in vitro

Values are mean ± S.E., number of animals, *P*-significance

	Non pregnant	Pregnant (fetus-carrying)	<i>P</i>
Threshold concentration	18.5 ± 4.5	3.0 ± 0.9	4 <0.05
Response	0.3 ± 0.1	2.5 ± 0.4	7 <0.01



ceptor studies the relationship between the postganglionic nerve terminals and their postsynaptic receptors can be more specifically studied. This has been done in human and rat myometrial strips (Nakanishi et al 1969; Hollingsworth 1975). In view of the previous structural and biochemical findings obtained from guinea pig studies, suggesting a pregnancy induced degeneration of the uterine adrenergic nerves during advancing pregnancy, the present study on the uterine adrenergic nerve-muscular relationship in non-pregnant and pregnant guinea pigs was undertaken for a functional comparison.

## MATERIALS AND METHODS

**Experimental procedure.** Female sexually mature virgin guinea-pigs (400–500 g b.wt.) were used. They were given a single s.c. dose of estradiol-17 $\beta$  dissolved in pea nut oil (100  $\mu$ g/250 g b.wt.) two days before sacrifice. 8, 4 and 1 day before killing, half the number of animals were given an i.v. injection of 6-OH-dopamine (6-OH DA) dissolved in ice cold saline containing 0.1 mg/ml of ascorbic acid in respective doses of 100 mg/kg, 100 mg/kg and 50 mg/kg. The animals were killed under a light ether anaesthesia and the uterine horns dissected out. From one horn some minute tissue pieces were rapidly taken for the fluorescence histochemical demonstration of adrenergic nerves according to the method of Falck and Hillarp (for details see Björkstrand et al 1972) and thereupon the whole uterine horn was quickly put into ice cold 0.4 N perchloric acid for the determination of the tissue content of noradrenaline according to the method of Bertler et al (1958) as modified by Häggendal (1963). The other uterine horn was put into ice cold Krebs-Ringer bicarbonate buffer (pH 7.4) immediately after the dissection and some minutes later myometrial tissue slices (about 6–9 mm in length, 2–3 mm in width and 0.2 mm in thickness) were prepared as described by McIlwain & Rodnight (1962) using a razor blade and a cold object slide. As judged from control sections examined in routine staining the slices obtained consisted of bundles of smooth muscle cells from the outer longitudinal smooth muscle layer. Myometrial tissue slices were also prepared from pregnant guinea-pigs after some minute pieces of uterine tissue had been taken for fluorescence histochemistry of adrenergic nerves. Most of the pregnant animals contained fetus(es) in both the uterine horns. Two animals carried fetuses in only one of the uterine horns. The time of pregnancy was about 60 days. It was estimated by measuring the crown rump length and weight of the fetus(es) (Draper 1920; Kaufmann 1969).

The myometrial strips were mounted between platinum electrodes (5 mm apart) in an organ bath containing 30 ml of Tyrode solution (NaCl 0.8, KCl 0.07, CaCl<sub>2</sub> 0.02, MgCl<sub>2</sub> 0.01, NaHCO<sub>3</sub> 0.1, NaH<sub>2</sub>PO<sub>4</sub> 0.005 and glucose 0.1%). The solution was maintained at 37°C and aerated with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After mounting the strips had to equilibrate in the organ bath for about 3 h before

experiments were undertaken. The tension of the myometrial strips was measured isometrically by strain gauge transducers and recorded on a Rikenscribe servo-recorder. The initial tension was 1 g. The strips then contracted spontaneously evoked by adding metacholine or bradykinin 3–5  $\mu$ g to the organ bath, raising the tension to 2–4 g. Electrical field stimulation: a Grass stimulator gave square pulses with a duration of 2 ms, a frequency of 1 Hz and of supramaximal voltage was used.

In every expt. a myometrial strip from a non-pregnant guinea-pig or from a non-pregnant guinea-pig with 6-OH DA and a strip from a non-pregnant guinea-pig were studied simultaneously mounted in the organ bath. In two expts. using animals with isolated uterine horns a strip from the empty (non-fetus-containing) horn and a strip from the contralateral fetus-containing horn from the same animal were studied in the same bath.

**Statistics.** Student's *t*-test was used. The 5% probability was accepted as significant.

**Drugs.** The following substances were used: estradiol-17 $\beta$ , 6-OH-dopamine, metacholine chloride, triacetate, noradrenaline bitartrate, dihydro- $\alpha$ -methansulphonate, propranolol hydrochloride, ethidine bisulphate, hexamethonium bromide, picrotoxin. The drugs were injected directly into the peritoneal cavity. Concentrations given are final bath concentrations of the substance.

## RESULTS

**Histochemistry.** In virgin animals only few adrenergic myometrial nerves as well as in the bundles of smooth muscle cells as around blood vessels in accordance with previous findings (1968; Thorbert et al 1977). However, no adrenergic myometrial nerves could be seen after the combined treatment of estradiol-17 $\beta$  and 6-OH DA in pregnant animals, whether the uterine horns contained fetuses or not.

**Tissue content of NA.** 6-OH DA produced a pronounced reduction of the endogenous noradrenaline measured per uterine horn about 95% compared to animals given only estradiol-17 $\beta$ .  $0.305 \pm 0.052$   $\mu$ g to  $0.015 \pm 0.001$   $\mu$ g/mg tissue,  $n=4$ ,  $P<0.001$ .

**Stimulation experiments.** Electrical field stimulation caused relaxation of the uterus from non-pregnant guinea pigs, reducing the tone from 1.4 to 1.4  $\pm$  0.1 g (mean  $\pm$  S.E.,  $n=14$ ), i.e. by 30% ( $P<0.001$ , paired comparisons). The responses were not affected by previous addition of hexamethonium 300  $\mu$ g/ml but were abolished by guanethidine 3  $\mu$ g/ml, propranolol 3  $\mu$ g/ml or tetrodotoxin 1  $\mu$ g/ml. In myo-

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uterus (Thorbert et al 1977). It should be inferred that in the guinea pig uterus there have recently been demonstrated populations of nerves which compared to the adrenergic nerves occur in a less amount and with a somewhat different intramural distribution and further contain intraneuronal peptides such as substance P or vasoactive intestinal polypeptide (VIP) (Alm et al 1977, 1978; Larsson et al 1977). The uterine functions mediated by these peptidergic nerves are however unknown. The unchanged field stimulation induced relaxation after the administration of hexamethonium indicate that the nerves are postganglionic adrenergic fibres. From a functional point of view this is important to strengthen as histochemical studies have demonstrated that the guinea pig uterus is supplied both with long and short adrenergic neurons with their ganglionic cell bodies located respectively at a distance from or very close (even intramurally) to the organ (Sjöberg 1968; Thorbert et al 1977). The complete blockade of the field stimulation induced response after the administration of propranolol suggests that the nerve induced relaxation is mediated via  $\beta$  receptors.

It has earlier been demonstrated that during advancing pregnancy the level of uterine adrenergic transmitter is highly reduced. At the time for parturition no adrenergic nerves can be demonstrated histochemically and almost no noradrenaline transmitter can be demonstrated by quantitative fluorometric analyses (Sjöberg 1968; Owman et al 1975; Thorbert et al 1979). The almost completely abolished axonal transmitter uptake mechanism (Alm et al 1979b), the absence of activity of the noradrenaline synthesizing enzyme tyrosine hydroxylase at term pregnancy (Alm et al 1979a) in combination with the occurrence of ultrastructural signs of adrenergic terminal degeneration during advancing pregnancy (Sporrøng et al 1978) suggest that the absence of uterine noradrenaline transmitter in the fetus-carrying horn is due to degeneration of the adrenergic nerve plexus. This may in the present study be verified functionally by the almost total absence of a field stimulation induced response in myometrial strips from fetus-carrying uterine horns. Further at the time for parturition the threshold concentration to get a noradrenaline induced response is lower and the responses induced are greater compared to strips from non-pregnant myometrial tissue. Thus in the fetus-carrying uterine horn the pregnancy induced adrener-

gic nerve degeneration is also accompanied by increased postsynaptic adrenoceptor sensitivity to the noradrenaline transmitter. This phenomenon could correspond to the well-known dense supersensitivity described in other organs after adrenergic nerve denervation (see review by Föllin & Trendelenburg 1972).

Also in the empty horn in unilateral pregnancy the level of noradrenaline transmitter was highly reduced at the time for parturition as from previous histochemical and quantitative fluorimetric studies (Owman et al 1975; Thorbert et al 1979). However in contrast to the fetus-carrying uterine horn the adrenergic nerve plexus in the empty horn and the cervix seemed to be structurally intact as the fluorescence microscopic pattern of uterine adrenergic nerve plexus was revealed in vitro incubation in the presence of a methylated noradrenaline and further there still seemed to be a substantial and effective axonal noradrenaline transmitter uptake mechanism at term pregnancy (Alm et al 1978b; Thorbert et al 1979). In the present material is small the findings in myometrial strips of empty uterine horns demonstrate a distinct relaxatory response induced by electrical field stimulation and a moderate relaxatory response after the administration of exogenous noradrenaline. On the contrary as from the contralateral fetus-carrying uterine horn no response could be evoked by field stimulation. Taken together these findings might favor the previous assumption (see above) of a more or less structurally intact adrenergic innervation of the empty uterine horn still able to evoke a field stimulation response. It should be inferred that the low share of the total neuronal noradrenaline accumulation to evoke a physiological response is very small (cf. Lundborg 1967). This may explain the present field stimulation induced response in strips of the empty horn having a very low transmitter level.

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# Barrier mechanisms for neurotransmitter monoamines in the choroid plexus

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LINDVALL, M. HARDEBO J. E. & OWMAN CH. Barrier mechanisms for neurotransmitter monoamines in the choroid plexus. *Acta Physiol Scand* 1980 108 215-221. Received 28 May 1979. ISSN 0001-6772. Departments of Histology and Neurology, University of Lund, Sweden.

Studies were performed on choroid plexuses from mouse and rat. The plexus tissue, sympathectomized to avoid interference from adrenergic nerves, is able to accumulate DOPA as well as NA and 5-HT against concentration gradients. Within the plexus tissue these compounds are metabolized by aromatic L-amino acid decarboxylase (MAO) and COMT, whose activities were determined radiochemically. On the basis of this and the fate of systemically administered L-DOPA and DA studied by fluorescence histochemistry it is suggested that the epithelium of the choroid plexus provides not only a structural but also an enzymatic barrier for transmitter amines. The enzymatic barrier for amine precursors is considerably less sufficient at this blood-CSF interphase. The trapping of monoamines resembles the enzymatic barrier present in the endothelial cells and pericytes at the microvascular level of the blood-brain interphase.

**Key words:** Choroid plexus, blood-CSF barrier, amines and amine precursors, MAO, COMT.

Exchange between the circulation and the brain cytochrome must be strictly regulated in order to maintain adequate function of the brain. In order to accomplish this, the interphase between the blood and brain intercellular fluid compartment is equipped with morphological barrier properties, located at the level of the endothelial cell lining in the blood vessels within the brain tissue (Reese & Karnovsky 1968; Westergaard & Brightman 1973; van Deursen & Brightman 1975). If substances are to pass beyond the choroid plexus, they would have to freely enter the brain parenchyma from the ventricular system. The barrier has a selective action so that several substances may pass the barrier of the luminal cellular lining of the brain vessels—i.e. the endothelial cells and pericytes—and the plexus epithelial cells with various types of transport mechanisms. This is the case for monoamine transmitters DA, NA, adrenaline, 5-HT in the direction from the brain to the circulation (Hardebo & Owman 1979) and for its inactive precursors e.g. L-DOPA. In both directions (Berthier et al. 1966; Wade & Katzman 1975;

Hardebo et al. 1979 for review; see Hardebo & Owman 1979b). Therefore, in order to assist in the barrier functions at the blood-brain interphase, these cells must possess an efficient intracellular breakdown mechanism for such substances. In the cerebral microvessels this breakdown is effectuated by enzymes present within the endothelial cells and pericytes, namely AAD and MAO leading to a conversion of these substances into inactive metabolites (for review; see Hardebo & Owman 1979b).

The present study was undertaken to elucidate the possible presence of a corresponding enzymatic barrier mechanism at the specialized component of the blood-brain interphase constituted by the choroid plexus. Since the microvessels in the plexus are porous in that they have fenestrated endothelium, which permit the ready passage of solutes, the

Abbreviations used: AAD, aromatic L-amino acid decarboxylase; BBB, blood-brain barrier; COMT, catechol-O-methyltransferase; CSF, cerebrospinal fluid; DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; 5-HT, 5-hydroxytryptamine; MAO, monoamine oxidase; NA, noradrenaline.





Fig. 1. Fluorescence photomicrographs of mouse choroid plexus and adjacent brain parenchyma. 160 (a) L-DOPA (50 mg/kg) following MAO inhibition. A diffuse fluorescence has accumulated in the plexus, and the very walls of the brain parenchyma are intensely fluorescent. (b) DA (500 mg/kg). An intense green fluorescence is found in the wall of plexus blood vessels and in adjacent connective tissue of the basement membrane of plexus epithelium and the brain parenchyma are equally non-fluorescent. (c) DA (500 mg/kg) following treatment with nialamide. An intense fluorescence is found also in the epithelium of the choroid plexus.

cyde reaction was that of the noradrenergic cells (Edviusson et al. 1974, 1975; Lindvall et al. 1978; Lindvall 1979), running in between the plexus epithelium and the wall of the underlying blood vessels. Mast cells, which in mice and rats contain IT visible by the formaldehyde method, were totally not found in the plexuses.

Following systemic administration of L-DOPA or a pronounced fluorescence microscopic changes detectable by the formaldehyde technique occurred in the choroid plexuses.

A green fluorescence seen after administration of DOPA was diffusely distributed in the vessel walls and in the epithelium. The intensity was low at the 50 mg/kg dose; it was enhanced at increasing doses and was high at 160 mg/kg. Pretreatment of the

animals with nialamide before the L-DOPA injection further increased the green fluorescence in the plexuses (Fig. 1a). Animals receiving the decarboxylase inhibitors, NSD-1015 or benserazide showed a slightly elevated green fluorescence compared to animals given L-DOPA alone though the distribution of the fluorophore in the plexus tissue was not different from that of the latter group of animals. Administration of DA also resulted in a green fluorescence of the choroid plexus, but the distribution was partly different from that obtained after treatment with L-DOPA. As for L-DOPA, DA accumulated in the wall of the blood vessels and in the adjacent connective tissue of the basement membrane. However only a weak diffuse fluorescence was found in the epithelium. Taking the fluorescence at the 100 mg/kg dose for comparison this was found to be distinctly less pronounced than the epithelial cell fluorescence induced by 50 mg/kg of L-DOPA. The vascular and subepithelial fluorescence was considerably enhanced with increasing doses of DA (Fig. 1b). When the DA treatment was combined with MAO inhibition a very bright fluorescence appeared also in the plexus epithelium (Fig. 1c).

**Quantitative estimations of enzyme activity.** The observations are summarized in Table 1. AAD activity was found in the choroid plexuses from both mouse and rat. Sympathetic denervation reduced the activity considerably in rat but not in mouse. Treatment of mice with the decarboxylase inhibitor benserazide almost abolished the AAD activity (Table 2). A substantial MAO activity was found in the plexus tissue: most of it remaining after sympathectomy. The dose of the MAO antagonist, nialamide used in the fluorescence histochemical studies (100 mg/kg) inhibited the enzyme activity almost completely (Table 2). The choroid plexus of rat also contained a high activity of COMT.

**Histochemistry of MAO.** Histochemical analysis on mice showed that the choroid plexus contained MAO as shown by an overall staining of the tissue but the rather low precision of the technique did not reveal any particular association of the formazan deposits to the various cellular components of the tissue. Nialamide-treated mice did not show any visible MAO activity in their choroid plexuses.

**Incubation of isolated choroid plexuses.** An accumulation of radioactivity was obtained in the choroid plexuses after incubation of tissues from the rat in the presence of varying concentrations of

barrier aspects are therefore in this context focussed on the epithelial lining of the choroid plexus

## MATERIAL AND METHODS

**Animals** The study was performed on choroid plexuses from adult animals: 250 female NMRI mice (25 g b wt) and 123 male Sprague Dawley rats (200–250 g b wt). The animals had free access to standard pellet food and water. In order to differentiate between neuronal and extraneuronal enzyme activity the perivascular sympathetic nerve plexus was eliminated by bilateral removal of the superior cervical ganglion under nembutal-ethyl ether anesthesia 10 days prior to the measurement in 45 mice and 76 rats. The mice were killed by decapitation where as the rats were killed under light ethyl ether anesthesia by perfusion through the left ventricle or the heart with 0.9% saline. After perfusion and/or decapitation of the animals the brains were immediately removed and the choroid plexuses from the lateral ventricles dissected out. In the mice in addition the plexus of the third ventricle as well as the brain (excluding the brain stem) were dissected out.

**Drug treatments** Groups of mice (6–10 in each group) received the following i.p. injections of drugs dissolved in 0.9% saline (the time intervals between two types of injections and between the last injection and decapitation are given within parentheses):

- I. Normal untreated control animals
- II. L. DOPA (Sigma) 10, 50 or 160 mg/kg (70 min)
- III. Nialamide (Pfizer) 100 mg/kg (60 min)
- IV. Nialamide 100 mg/kg (60 min) and L. DOPA 50 mg/kg (70 min)
- V. Ro 4-4602 (benserazide, Roche) 50 mg/kg (30 min) or NSD 1015 (Smith, Kline and French) 100 mg/kg (60 min)
- VI. Ro 4-4602 50 mg/kg (30 min) and L. DOPA 50 mg/kg (70 min)
- VII. NSD 1015 100 mg/kg (60 min) and L. DOPA 50 mg/kg (20 min)
- VIII. DA (dopamine hydrochloride, Sigma) 100 or 500 mg/kg (20 min)
- IX. Nialamide 100 mg/kg (60 min) and DA 100 or 500 mg/kg (70 min)

**Fluorescence histochemistry** The brain preparations were frozen in a propane-propylene mixture at the temperature of liquid nitrogen, freeze-dried and treated in gaseous formaldehyde (1 h at 80°C) according to the Falck-Hillarp method, embedded in paraffin *in vacuo* and sectioned at 6  $\mu$  thickness for fluorescence microscopy (for details, see Björklund et al. 1977).

**Chemical determination of enzyme activity** The choroid plexuses were homogenized in 70–60  $\mu$ l of 10 mM sodium or potassium phosphate buffer (pH 6.5–7.0) and aliquots were taken for radiometric estimation of AAD and MAO activity as described by Fonnum (1976). COMT activity (not measured in mice) was estimated according to the method described by Axelrod (1962). Choroid plexuses from 5 mice or rats were pooled for each determination of AAD/MAO and COMT activity respectively. Each enzymatic assay was linear with respect to protein concentration and incubation time and performed at 37°C conditions. Tissue protein was determined according to the method of Lowry et al. (1951).

**Histochemistry of MAO** The tetrazolium salt of Glensner et al. (1957) was used for cellular localization of MAO in mice. Cryostat sections (20  $\mu$ m thick) of brain tissue containing the choroid plexus were fixed for 30–45 min at 37°C in phosphate buffer (pH 7.4) containing tryptamine (Regis) as substrate and tetrazolium salt as the tetrazolium salt. Tissues from normal mice from mice given 100 mg/kg of the MAO inhibitor nialamide 1 h before decapitation were used. The controls were obtained by omitting the substrate (tryptamine) from the incubation solution.

**Incubation of isolated choroid plexus** Choroid plexuses from sympathectomized rats were plexuses were transferred to incubation vials (ice-cold Krebs Ringer buffer solution (see mM) NaCl 118, KCl 4.5, CaCl<sub>2</sub>  $\times$  2H<sub>2</sub>O 2.5, MgCl<sub>2</sub> 1.0, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.0 to which was mg/ml glucose and 0.1 mg/ml ascorbic acid. The solution was continuously aerated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The vials were placed in an incubator allowed to equilibrate for 20 min at 37°C before which was started by adding either NA at  $3 \times 10^{-6}$  and 10 $^{-5}$  M, 9 L Cl/mmol New England 5-HT (L. 5-HT-G-3H creatinine sulphate M M 8 Cl/mmol Radiochemical Centre Amersham DOPA (L. DOPA 2,3-<sup>3</sup>H  $3 \times 10^{-6}$  and  $10^{-5}$  M 43 Cl Radiochemical Centre Amersham) in the solution lower concentrations tested roughly correspond to normal circulating levels of NA, 5-HT and DOPA (Hardebo & Owman 1979b) and the higher to those used in the circulation during stress and certain pathological conditions and for DOPA in the medical treatment of Parkinson's disease. The tissue preparations were incubated in the presence of these compounds for 15 min at 37°C. The incubation was terminated by transferring the vials into ice-cold buffer solution. The plexus was washed twice in isotope-free cold buffer each for 15 min. In order to further characterize the uptake of the drugs were also incubated in the presence of reserpine (10 $^{-6}$  M) and at 0°C. This characterization was performed at concentrations corresponding to physiological levels in the circulation. In the CSF these concentrations are lower.

**Measurement of radioactivity** After incubation choroid plexuses were weighed and transferred to 15 ml scintillation vials. They were solubilized in 0.5 ml of 5% (Packard) and liquid ventilation counting was performed in 10 ml Instagel (Packard) as were appropriate (2–5  $\mu$ l) of the incubation medium. Quench correction were obtained according to conventional principles.

**Statistics** Differences between mean values were analyzed with the Student's *t* test for unpaired data.

## RESULTS

**Histochemistry of DOPA and DA** The choroid plexus was easily recognized in brain tissue from untreated animals by its slight green background fluorescence. The only specific fluorescence in the plexuses induced by the



Fluorescence photomicrographs of mouse choroid plexus and adjacent brain parenchyma. 160 (a) L-DOPA (50 mg/kg) following MAO inhibitor. A diffuse fluorescence has accumulated in the plexus, and the walls of the brain parenchyma are intensely fluorescent. (b) DA (500 mg/kg). An intense green fluorescence is found in the wall of plexus blood vessels and in connective tissues of the basement membrane. (c) DA (500 mg/kg) following MAO inhibitor. An intense fluorescence is found also in the epithelium of the choroid plexus.

the reaction was that of the noradrenergic system (Edvasson *et al.* 1974, 1975; Lindvall *et al.* 1979) running in between the plexus stroma and the wall of the underlying blood vessels. Mast cells, which in mice and rats contain visible granules, were not found in the plexuses.

Following systemic administration of L-DOPA or dopamine fluorescence microscopic changes observable by the formaldehyde technique occurred in the choroid plexuses.

Green fluorescence seen after administration of L-DOPA was diffusely distributed in the vessel walls and the epithelium. The intensity was low at the 50 mg/kg dose; it was enhanced at increasing doses and was high at 160 mg/kg. Pretreatment of the

animals with nialamide before the L-DOPA injection further increased the green fluorescence in the plexuses (Fig. 1a). Animals receiving the decarboxylase inhibitors NSD-1015 or benserazide showed a slightly elevated green fluorescence compared to animals given L-DOPA alone, though the distribution of the fluorophore in the plexus tissue was not different from that of the latter group of animals. Administration of DA also resulted in a green fluorescence of the choroid plexus, but the distribution was partly different from that obtained after treatment with L-DOPA. As for L-DOPA, DA accumulated in the wall of the blood vessels and in the adjacent connective tissue of the basement membrane. However, only a weak, diffuse fluorescence was found in the epithelium. Taking the fluorescence at the 100 mg/kg dose for comparison, this was found to be distinctly less pronounced than the epithelial cell fluorescence induced by 50 mg/kg of L-DOPA. The vascular and subepithelial fluorescence was considerably enhanced with increasing doses of DA (Fig. 1b). When the DA treatment was combined with MAO inhibition, a very bright fluorescence appeared also in the plexus epithelium (Fig. 1c).

**Quantitative estimations of enzyme activity.** The observations are summarized in Table 1. AAD activity was found in the choroid plexuses from both mouse and rat. Sympathetic denervation reduced the activity considerably in rat but not in mouse. Treatment of mice with the decarboxylase inhibitor benserazide almost abolished the AAD activity (Table 2). A substantial MAO activity was found in the plexus tissue, most of it remaining after sympathectomy. The dose of the MAO antagonist nialamide used in the fluorescence histochemical studies (100 mg/kg) inhibited the enzyme activity almost completely (Table 2). The choroid plexus of rat also contained a high activity of COMT.

**Histochemistry of MAO.** Histochemical analysis on mice showed that the choroid plexus contained MAO as shown by an overall staining of the tissue but the rather low precision of the technique did not reveal any particular association of the formazan deposits to the various cellular components of the tissue. Nialamide-treated mice did not show any visible MAO activity in their choroid plexuses.

**Incubation of isolated choroid plexus.** An accumulation of radioactivity was obtained in the choroid plexuses after incubation of tissues from the rat in the presence of varying concentrations of



Table 1 Activities of the enzymes aromatic L-amino acid decarboxylase (AAD), monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) in the choroid plexuses from untreated and SyX pathectomized (SyX) mouse and rat

SyX was performed by bilateral removal of the superior cervical ganglion 10 days prior to determination. Blanks subtracted. Mean values  $\pm$  S.E.M. Number of determinations within parenthesis. Statistical evaluation according to Student's *t*-test.  $0.001 < P < 0.01$  n.s. is non-significant.

Animal	Plexus tissue analyzed	AAD nmol/mg prot/h	MAO nmol/mg prot/h	COMT nmol/mg prot
Mouse	Intact	1.00 $\pm$ 0.11 (10)	123.1 $\pm$ 17.7 (10)	
	SyX	0.96 $\pm$ 0.11 (5) n.s.	83.7 $\pm$ 15.0 (5) n.s.	
Rat	Intact	1.99 $\pm$ 0.25 (4)	66.7 $\pm$ 3.0 (3)	1.91 $\pm$ 0.17
	SyX	0.5 $\pm$ 0.15 (3)	—	—

radioactive L-NA, L-5-HT or L-DOPA as evidenced by a higher radioactivity than in the incubation medium (Table 3). It should be noted that this represents the net accumulation of radiolabelled compound as a result of uptake and subsequent loss during the washing step. The accumulation was significantly reduced by incubation at 0°C and by blockade of Na<sup>+</sup>/K<sup>+</sup>-dependent ATPase activity with ouabain (Table 3).

## DISCUSSION

The histofluorescence analyses and the incubation experiment showed that the choroid plexus is able to take up L-DOPA against a concentration gradient involving an energy requiring mechanism. The process may thus be similar to that found for the capillary endothelium in the brain (see Hardebo & Owman 1979b). Inhibition of the decarboxylation step in the formation of DA from L-DOPA increases the circulating levels of L-DOPA which was reflected in a higher fluorescence in the choroid plexus tissue of animals given L-DOPA and pretreated with

benserazide or NSD 1015 supporting the idea that amino acid is indeed taken up by the plexus. The observation of a slightly higher plexus fluorescence when L-DOPA was given together with MAO inhibitor—in a dose that efficiently blocks MAO in the plexus—suggests that a minor amount (reflected only in a minor fluorescence increase) of the fluorogenic material in the plexus is metabolized by MAO. This would imply that a small part of the L-DOPA taken up has been decarboxylated to DA through the activity of (denervation-resistant) AAD found in the plexus. It should be pointed out, however, that the decarboxylation capacity is by far higher in the vascular endothelium of the brain parenchyma where it appears to constitute an important component in the enzymatic BBB mechanism (for review see Hardebo & Owman 1979b). When DA is given, an intense green fluorescence was noted in the wall of the plexus vasculature and also beyond the vessel wall in the connective tissue. However, the epithelium contained only small amounts of amine which would indicate that the amine

Table 2 Activities of L-amino acid decarboxylase (AAD) and monoamine oxidase (MAO) in the choroid plexus before and after treatment with the AAD inhibitor benserazide (50 mg/kg i.p. 30 min) or the MAO inhibitor nialamide (100 mg/kg i.p. 60 min)

Mean values  $\pm$  S.E. number of determinations within parenthesis. Student's *t*-test.  $P < 0.001$ .

Enzyme	Treatment		
	Control (nmol/mg prot/h)	Benserazide (nmol/mg prot/h)	Nialamide (nmol/mg prot/h)
AAD	1.78 $\pm$ 0.11 (5)	0.07 $\pm$ 0.01 (6)*	0.9 $\pm$ 0.2 (6)
MAO	153.8 $\pm$ 19.9 (5)	15.8 $\pm$ 3.4 (6)	83 $\pm$ 0.77 (6)

3 Net accumulation of radiomethylty (tissue/medium ratio) following incubation of sympathetic plexus tissue in the presence of either  $^3\text{H}$ -L-NA,  $^3\text{H}$ -5-HT or  $^3\text{H}$ -DOPA at two concentrations  $37^\circ\text{C}$  and the effect of low temperature or ouabain

med	Concentration (M)	$17^\circ\text{C}$	$37^\circ\text{C}$	Ouabain $10^{-6}$ M	Concentration (M)	$37^\circ\text{C}$
A	$3 \times 10^{-6}$	$4.46 \pm 0.37$ (6)	$0.52 \pm 0.13$ (6)	$1.47 \pm 0.19$ (4)	$1 \times 10^{-6}$	$2.91 \pm 0.37$ (5)
	$1 \times 10^{-6}$	$5.60 \pm 0.44$ (6)	$0.41 \pm 0.10$ (6)	$1.91 \pm 0.29$ (4)	$1 \times 10^{-6}$	$3.23 \pm 0.16$ (4)
	$3 \times 10^{-6}$	$3.05 \pm 0.33$ (6)	$0.83 \pm 0.26$ (6)	$1.21 \pm 0.17$ (4)	$1 \times 10^{-6}$	$1.86 \pm 0.19$ (4)

degree has entered the epithelium, or that it is efficiently degraded to non-fluorescent metabolites. The latter alternative is supported by a MAO activity (found histochemically and only in the plexus tissue (see also Landvall & al 1979). It is also supported by the considerable increase in fluorescence intensity when DA was after MAO inhibition (although a component of this increment is probably the higher concentration of circulating DA due to prevention of catabolism in peripheral tissues). That amines are only taken up into the plexus epithelium was shown in the incubation experiments, supporting the findings by Tochino & Schanzer (1965). The concentration of neurotransmitter monoamines and their precursors in the brain extracellular compartment has to be controlled within narrow limits in order to maintain an adequate function of the neuron systems. One aspect of this is the lateral exchange across the blood-brain interface (Wade & Katzman 1973; Hardebo & Owman 1979b), this also involves transport processes in the choroid plexuses, which constitute specialized regions of the cerebrovascular system. The choroid plexuses represent the main source for the formation of CSF (Davson 1967; Dobrman 1970; Caerr 1973; Polley 1975). The plexuses receive a developed supply of adrenergic sympathetic innervation (Edvinsson et al 1974, 1975; Landvall et al 1978; Landvall 1979) which have an inhibitory effect on the production of CSF (Landvall et al 1978). In the course of the histochemical studies on the enzymatic BBB for certain monoamines and their immediate precursor amino acids (Bertler et al 1964) it was found that the fluorogenic amino acids and amines to varying extent accumulated in the choroid plexus. The findings of a sympathetic influence on choroid plexus functions have initiated a renewed interest in the handling of neurotransmitters and related amines in the choroid plexus, and

its position in the barrier system for such compounds at the interphase between the blood and CSF.

Blood-borne molecules are restrained from entering the CSF at the level of continuous apical bands of tight junctions surrounding and sealing the epithelial cells (see Brightman 1975). The underlying capillary endothelium functionally resembles the leaky regions of the BBB, molecules and ions may pass across the cellular fenestra, between adjacent endothelial cells which are connected only by the discontinuous type of tight junctions, and possibly also by vesicular transendothelial transport (Brightman 1975).

In brain microvessels, and also in brain arterioles and pial vessels, an enzymatic barrier mechanism for these monoamines and also for their immediate precursors exist (Bertler et al 1966)—besides the morphological barrier in parenchymal vessels represented by the continuous tight junctions between adjacent endothelial cells, in combination with paucity of micropinocytotic vesicles (Reese & Karnovsky 1967). The enzymatic barrier involves an uptake into not only the endothelium but also the smooth muscle cell layer of the vessel walls followed by breakdown of the amine by MAO and COMT; the precursor is first decarboxylated within the endothelium to the corresponding amine (see Hardebo & Owman 1979b). The presence of a similar enzymatic barrier to the amines at the blood-CSF interphase is suggested by the present findings of an energy-dependent amine uptake (see also Tochino & Schanzer 1965) and a subsequent degradation within the choroid plexus.

COMT is functionally related to the adrenergic receptor mechanism and has an extraneuronal localization in the autonomic innervation apparatus. Such localization of COMT can thus be assumed also in the choroid plexus. The MAO activity of the choroid plexus is both of the A and B

type (Lindvall & Owman 1979) there is reason to believe that the MAO A activity is primarily related to the sympathetic nerves present in the plexus. In order to eliminate any error related to the presence of sympathetic nerves in the plexus tissue in the determinations of the amine uptake capacity sympathetic denervation was carried out beforehand in the incubation experiments. Even though the perivascular nerve plexuses in the cerebrovascular system are traditionally not included among the components constituting the enzymatic BBB it is likely that the efficient neuronal transmitter inactivation mechanism represented by the adrenergic nerves will nevertheless assist in impeding the passage of sympathomimetic amines also at the level of the choroid plexus. Apart from being involved in an enzymatic barrier mechanism functional studies have suggested that MAO participates in the regulation of an inhibitory tone—in which the sympathetic nerves are involved—on the CSF production (Lindvall & Owman 1979).

The enzymatic mechanism at the blood-CSF interphase may function for the passage of monoamines across the choroid plexus in both directions i.e. to prevent the passage of circulating monoamines into the CSF and thereby the brain extracellular fluid compartment, and also to assist in the metabolism of amines present in the CSF. The metabolites formed by the enzymatic degradation of neurotransmitter monoamines such as homovanillic acid and 5-hydroxyindolic acid are excreted from the CSF to the circulation across the choroid plexus (Neff et al 1967; Ashcroft et al 1968; Cserr & van Dyke 1971; Bass & Lundborg 1976).

Although L-DOPA is actively taken up into the choroid plexus epithelium (after its local passage through the capillary walls) the subsequent decarboxylation is only slight in comparison with that occurring in the endothelium of the BBB. Other enzymes might be involved such as transaminase or COMT though this is not consistent with the intense epithelial fluorescence seen after L-DOPA administration since the products formed (dihydroxyphenylacetic acid and 3-O-methyl DOPA) are not fluorogenic in the formaldehyde reaction (see Björklund et al 1972). It is therefore probable that the uptake of L-DOPA is primarily a reflection of the transport into the choroid plexus of amino acids participating in the protein synthesis. The choroid plexus thus seems to constitute an inefficient bar-

rier for L-DOPA in a similar way as the plexus (Hardebo & Owman 1979b).

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# Characterization of the *in vitro* uptake of monoamines into brain microvessels

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The ability of brain microvessels to take up and metabolize transmitter monoamines (DA, NA, A and 5-HT) was studied in tissues from several species (rat, rabbit, guinea-pig, cat, dog, baboon, man) *in vitro*. Following incubation, slices were analyzed by fluorescence histochemistry and isolated microvessels by measurement of radioactivity from tritiated amine. Provided the MAO activity is inhibited, strong accumulation, similar for all animals tested, occurred in the wall (endothelial cells and pericytes) of capillaries and venules—but not in larger vessels—of all species. The degree of amine uptake and accumulation and the conditions under which it was inhibited suggested that it reflected a saturable, energy-dependent process with characteristics of both the extraneuronal and neuronal type of uptake processes. The mechanism may serve as an inactivation of transmitter monoamines at the blood-brain interface, thereby assisting in the control of transmitter levels in the cerebral extracellular compartment.

**Key words:** Blood-brain interface, brain microvessels, neurotransmitter monoamines, MAO.

Existence of a morphologic barrier between the blood and brain parenchyma is well documented (review see Rapoport 1976). Another aspect of barrier function is the presence of an enzymatic blood-brain barrier for monoamine precursors, such as 3,4-dihydroxyphenylalanine (L-DOPA) and hydroxytryptophan, first shown by Bertler *et al.* (1966) using fluorometric assay and fluorescence histochemical techniques. Systematically administered L-DOPA and L-5-HTP are taken up into the endothelial cells and pericytes of cerebral microvessels and is subsequently decarboxylated to the corresponding amine, i.e. dopamine (DA) and 5-hydroxytryptamine (5-HT). Using the same techniques it has been found that only minor amounts of circulating neurotransmitter monoamines, such as DA, noradrenaline (NA), adrenaline (A) and 5-HT accumulate within the endothelial cells and pericytes of cerebral microvessels (Hardebo *et al.* 1979): the bulk of monoamines is prevented from leaving the brain circulation already at the luminal membrane of the

endothelial cells (Bertler *et al.* 1966, Hardebo *et al.* 1979a). Evidence has been presented for the existence for an enzymatic blood-brain barrier not only for the monoamine precursors but also for the monoamines themselves: they are rapidly broken down by monoamine oxidase (MAO) present in the endothelial cells and pericytes of the brain microvessels (Bertler *et al.* 1966, Spector, Baird-Lambert & Liu 1977, Hardebo *et al.* 1977 and 1979b) and in addition by MAO and catechol-O-methyl transferase (COMT) in the smooth muscle cell layer of blood vessels and brain parenchymal arterioles (Hardebo *et al.* 1979b). On the other hand, little is known about the clearance of neurotransmitter monoamines in direction from the brain parenchyma to the circulation. It can be assumed that the local presence of a sufficient excretory mechanism into the cerebral microcirculation—along with the reuptake into the aminergic nerve terminal and the clearance from the cerebrospinal fluid—will assist in the control of the level of the amine in the brain extracellular compartment. In the present study a

brain microvascular uptake of various neurotransmitter monoamines is demonstrated and characterized

## MATERIAL AND METHODS

**Animals.** The study was performed on adult animals of either sex, namely 64 Sprague Dawley rats, 79 albino rabbits, 7 guinea-pigs, 3 cats, 4 dogs, 4 baboons and brain tissue from 5 adult female and male patients. The laboratory animals had free access to food and water. These animals were killed under Nembutal anesthesia by perfusion of the vascular system with 0.9% saline to eliminate any error caused by the presence of blood cells. The brains were dissected out and kept in Krebs Ringer buffer solution on ice (for composition see below) or during preparation of microvessel fractions in cold phosphate buffer. The human tissue (macroscopically intact frontal and temporal cortex) was obtained during neurosurgical lobe resection operations; the tissue was immediately placed in ice-cold buffer solution and transported on ice to the laboratory (within 30 min).

**Incubation of the slices.** Approximately 1 mm thin slices of the parietal cortex (frontal and temporal cortex in man), caudate nucleus, cerebellum, hypothalamus (at the level of the median eminence), spinal cord and heart from the various animals were cut with a razor blade and transferred to incubation vials containing ice-cold Krebs-Ringer buffer solution. The buffer solution had the following composition (mM): NaCl 118, KCl 4.5,  $\text{CaCl}_2 \times \text{H}_2\text{O}$  5,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  10,  $\text{NaHCO}_3$  25,  $\text{KH}_2\text{PO}_4$  1.0 to which was added 1 mg/ml glucose, 0.1 mg/ml ascorbic acid and 0.05 mg/ml EDTA.

The vials were placed in an incubation bath at 37°C for preincubation during 20 min in the presence of the MAO inhibitor, nialamide (10<sup>-4</sup> M) followed by incubation during 70 min after the addition of various doses (1, 10 or 100 µg/ml) of NA, A, DA or 5-HT. In addition, slices from the parietal cortex of rat were incubated in the presence of several increasing doses (1, 5, 10, 20, 40 or 100 µg/ml) of NA in an attempt to find an upper limit for the uptake process. The buffer solution was continuously aerated with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  giving pH of about 7.4 and a  $p\text{O}_2$  around 200 mmHg (as measured on an MRK II blood gas analyzer, Radiometer, Copenhagen). Slices from the parietal cortex (frontal and temporal cortex in man) were also incubated with NA, A, DA or 5-HT (1 or 10 µg/ml) in the absence of nialamide. Control slices were incubated without addition of any drugs.

In order to further characterize the uptake, the incubations of the parietal cortex (frontal or temporal cortex in man) with monoamines were also performed in the presence of either cocaine (10<sup>-6</sup> or 10<sup>-4</sup> M), desmethylnorpramine (10<sup>-6</sup> or 10<sup>-4</sup> M), phenoxybenzamine (10<sup>-6</sup> or 10<sup>-4</sup> M), oestradiol-17 $\beta$  (10<sup>-6</sup> or 10<sup>-4</sup> M), theophylline (10<sup>-6</sup> M), ouabain (10<sup>-6</sup> or 10<sup>-4</sup> M) or dinitrophenol (5  $\times$  10<sup>-6</sup> or 10<sup>-2</sup> M). These compounds were added to the bath at the start of the preincubation together with nialamide. Incubation was also performed in a low Na/high K milieu (in the buffer solution KCl substituted for all NaCl) and under a combination of anoxic conditions (continuous

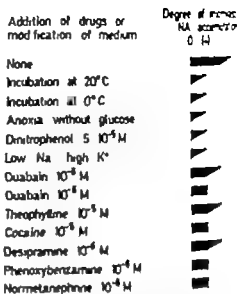


Fig. 1. Accumulation of NA fluorophore in the rat microvessels in rat cortical slices incubated in the presence of 10 µg/ml noradrenaline following 40 min incubation of 10<sup>-4</sup> M nialamide and processed according to the dihydro histofluorescence method. The degree of vascular fluorescence intensity above the differential background fluorescence is expressed by the tapering part of the bars indicating intermediate values.

bubble with 95%  $\text{N}_2$  and 5%  $\text{CO}_2$ ) and deplete energy substrate (glucose) and also at 20°C and 0°C.

**Fluorescence microscopy.** Following the incubation the various tissue pieces were frozen to the temperature of liquid nitrogen and further processed for fluorescent monoamine histochemistry according to the von Falck Hillarp method (Björklund, Falck & Owman, 1972). The paraformaldehyde used in the histochemical procedure had previously been equilibrated with air of 100% humidity. The formaldehyde-induced fluorescently visible fluorophores of NA, A and DA are indistinguishable in the sense that they have the same spectral characteristics and exhibit a green light under the optical conditions used, whereas 5-HT exhibits a yellow, more or less fading light (Björklund et al. 1972). Fluorescence microscopy was carried out on 6 µm thick sections using a fluorescence microscope equipped with an Osram 700 mercury lamp and Schott BG 12 and OG 490 primary and secondary filters, respectively. The relative fluorescence intensity in the microvessel walls was estimated according to an arbitrary scale (see Fig. 1) using the same preparations. The different preparations to be compared after incubation at the various conditions were always freeze-dried and further processed simultaneously.

**Isolation of cerebral microvessels.** Whole brain of rat and rabbit were used. After removal of the meninges in ice-cold 67 mM phosphate buffer (pH 7.4) the out the procedure (see Harlebo et al. 1979). The meninges, including the pia mater and its

fully torn off and the choroid plexuses were  
 11. Whole matter including the whole brain stem,  
 12. sliced away. Tissue obtained from 4-6 rats or 2-3  
 13. was chopped into a razor blade, and then gently  
 14. stripped by some 10 strokes up and down through  
 15. Serate 10-20 ml plastic syringes equipped with  
 16. (pore size 1000  $\mu$ m, 500  $\mu$ m and 200  $\mu$ m, respec-  
 17. tively) in their open cut end. The material was  
 18. easily homogenized by hand into a loosely fitting  
 19. side in smooth glass tube (0.1 mm clearance)  
 20. isogenic as washed through one nylon sieve  
 21. 1  $\mu$ m pore size. The material remaining on the  
 22. for extensive washing was re-homogenized and  
 23. 3-4 times through the same carefully washed  
 24. by tissue passing these sieves was collected and  
 25. through another sieve with 75  $\mu$ m pore size. The  
 26. remaining on this sieve after washing was re-  
 27. moved and re-moved in the same carefully washed  
 28. by tissue fraction remaining on the last sieve after  
 29. washing consists of capillaries, venules and a  
 30. or vessels, whereas most of the larger vessels and  
 31. branching off into clusters of small vessels, as well  
 32. small clusters of neurons and glia, remains on the  
 33. sieve (Härdéus et al. 1979a). Neuronal glia, small  
 34. and segments and subcellular fragments pass the  
 35. sieve. The capillary fraction is pure with regard to  
 36. and is easily contaminated with few glial elements  
 37. into the vessel wall (Härdéus et al. 1977). The yield  
 38. of vessels is less than 1/1000 of the original wet  
 39. of the grey matter. Brandel, Mezeas & Carbone  
 40. have shown that a mixed fraction of astrocytes  
 41. prepared under similar conditions is metabolically

42. **Isolation of isolated microvessels.** The microvessel  
 43. fraction was transferred to incubation vials containing  
 44. Krebs-Ringer buffer solution (composition as  
 45. 1. The vials were placed in an incubation bath and  
 46. 2. equilibrated for 20 min at 37°C before incubation  
 47. 3. back was started by adding  $^3$ H-NA to the solution.  
 48. 4. The preparations were incubated for 15 min at  
 49. 5. temperature of 37°C. The buffer solution was continuously  
 50. 6. stirred with 99%  $O_2$  and 1%  $CO_2$ . The incubation was  
 51. 7. ended by transferring the tissue into ice-cold buffer.

52. The microvessel fraction was collected by cen-  
 53. 54. trifugation at 4°C at 110 g and washed twice in isotonic-free  
 55. 56. buffer for each 40 min.

57. In order to further characterize the uptake, microvessel  
 58. 59. fractions were also incubated in the presence of ouabain  
 60. 61. (1  $\mu$ M), under combined normoxia and absence of glucose  
 62. 63. at 37°C.

64. **Assay of radioactivity.** After incubation, the  
 65. 66. vessel samples are weighed and transferred to  
 67. 68. scintillation vials. They are solubilized in 0.5 ml  
 69. 70. of (Packard), and liquid scintillation counting was  
 71. 72. performed in 10 ml bedged (Packard), as are approx-  
 73. 74. imately (25  $\mu$ l) of the incubation medium. Quench  
 75. 76. correction is obtained according to conventional pro-

77. 78. **Drugs:** Dopamine hydrochloride (Sigma); -noradren-  
 79. 80. aline monohydrochloride (Sigma); L-adrenaline bitar-  
 81. 82. tate (Sigma); 3-hydroxytryptamine creatinine hy-  
 83. 84. drochloride (Sigma); nialamide hydrochloride (ACO), om-

85. 86. hal (Sigma), 1,4-diaminophenol (Chroma), thioraphiline  
 87. 88. (Sigma), desmethyl imipramine (Desipramine Geigy),  
 89. 90. phenosybenzamide hydrochloride (Dobutamine Smith  
 91. 92. Kline and French), nialamide (Pfizer) and L-norepineph-  
 93. 94. rine  $^3$ H (New England Nuclear 9.1 Ci/nmol).

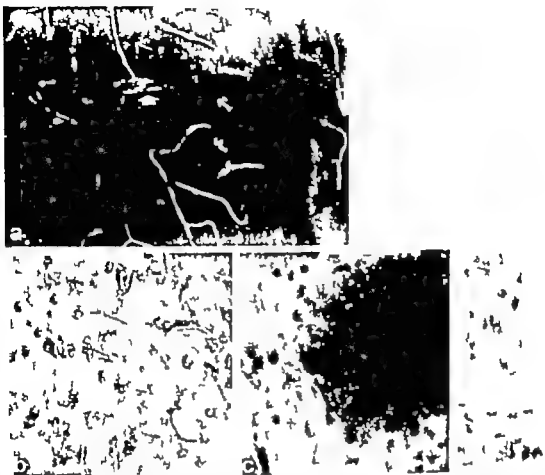
95. **Statistics.** Mean values were compared according to the  
 96. 97. Student's *t*-test for unpaired data.

## RESULTS

98. **Incubation of tissue slices.** Fluorescence micro-  
 99. 100. scopy of the various brain regions from control  
 101. slices incubated in buffer solution alone showed a  
 102. dense network of delicate catecholamine-containing  
 103. nerve terminals emitting a green fluorescence in  
 104. the cerebellum only a small number of isolated  
 105. green-fluorescent axons were present. The paren-  
 106. chymal blood vessels were equipped with varying  
 107. amounts of sympathetic nerves. In the heart tissue  
 108. adrenergic nerves were seen in the myocardium and in  
 109. association with the blood vessels. All tissues  
 110. showed a slight diffuse non-specific greenish  
 111. background fluorescence. The vessel wall proper  
 112. was in all regions essentially non-fluorescent ex-  
 113. cept for the intense autofluorescence of the internal  
 114. elastic membrane of larger pial arteries.

115. After incubation with the various monoamines  
 116. an uptake (as reflected in an accumulation of  
 117. fluorophore well above the diffuse background  
 118. fluorescence) was seen in the brain microvessel  
 119. walls (endothelial cells and pericytes of capillaries  
 120. and small veins) in all species studied. The intensity  
 121. of the general background fluorescence was also  
 122. increased. No clearcut accumulation above the dif-  
 123. fuse background fluorescence had occurred in the  
 124. walls of the brain parenchymal arterioles or heart  
 125. vessels. Possibly a slight uptake and accumulation  
 126. was seen in the wall of pial arteries. Inhibition of  
 127. MAO by nialamide clearly enhanced the accumula-  
 128. tion of the monoamines in the microvessel wall, as  
 129. evidenced by a stronger fluorescence intensity in  
 130. relation to the background fluorescence. At an iden-  
 131. tical concentration in the incubation bath DA, NA  
 132. and A all induced a similarly strong fluorescence in  
 133. the microvessel wall, whereas the fluorescence in-  
 134. duced by 5-HT was weaker. This discrepancy in  
 135. fluorescence intensity is at least partly explainable  
 136. by the varying fluorescence yield in the standard  
 137. formaldehyde reaction (Björklund et al. 1977). An  
 138. upper limit of fluorescence intensity in brain mi-  
 139. crovessel walls could be disclosed when the tissue





**Fig. 1** Fluorescence photomicrographs of brain tissue slices following incubation in the presence of catecholamine. (a) Human temporal cortex  $\times 90$  (a) Intense fluorescence in microvessel walls but no accumulation in arterioles (arrow) after incubation with  $10 \mu\text{g/ml}$  norepinephrine in the presence of ouabain  $10^{-4} \text{ M}$ . The degree of norepinephrine accumulation is markedly reduced. (c) During hypoxia only very little norepinephrine accumulates in the microvessel walls. (d) Rat parietal cortex. (d) Intense fluorescence in the walls of capillaries and venules (v) but not in arterioles (arrow) after incubation with  $10 \mu\text{g/ml}$  desipramine. (e) Desipramine ( $10^{-4} \text{ M}$ ) reduces the microvascular accumulation so that only the more voluminous vessels are clearly visible  $\times 75$ .

slices pretreated with nialamide were incubated in the presence of increasing concentrations of NA. A maximum fluorescence was seen at the  $20 \mu\text{g/ml}$  concentration. It has been shown in model experiments that there is a linear relationship between fluorescence intensity and the catecholamine concentration. Due to quenching of the fluorescence at high catecholamine concentrations a further increase in the concentration above a certain level gives an unchanged or even slightly diminished intensity (Ritzén 1966, Jonsson 1971). However, our finding most probably reflects an upper limit for accumulation in the microvessel wall rather than being the result of quenching, which occurs only at very high local concentrations.

The inhibitory influence on the uptake of NA by various drugs or by modification of the metabolism is listed in Fig. 1 and illustrated in Fig. 2. The effect of inhibition was the same for the other  $\alpha$ -aminines studied except for 5-HT which was resistant to desipramine treatment.

**Incubation of microvessels.** An uptake and accumulation of radioactivity was obtained in the microvessel fraction after incubation in the presence of tritiated NA, as evidenced by a considerable higher radioactivity than in the incubation medium (Fig. 3). The uptake was significantly reduced by blockade of  $\text{Na}^+/\text{K}^+$ -dependent ATPase with ouabain by incubation under anaerobic conditions in the absence of glucose or (Fig. 3).

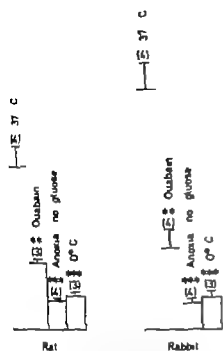


Fig. 4. Trans/medium ratio (T/M: mg tissue per  $\mu$ l) following incubation of isolated cerebral microvessels in the presence of  $^3$ H-NA under control conditions (37°C), and in the presence of ouabain ( $10^{-5}$  M), anoxia and deprivation of glucose, or at 0°C. Values are means  $\pm$  S.E., number of animals within parentheses. Control vs. ouabain according to Student's *t*-test.  $0.001 < p < 0.01$  ( $n=6$ ).

### DISCUSSION

The present study provides evidence for the existence in various species including man of a saturable, energy-dependent uptake mechanism for transmitter monoamines in the endothelial and pericytes of brain microvessels in contrast to the endothelium of brain parenchymal arteries. For comparison, in e.g. the erythrocytes amine uptake occurs by facilitated diffusion (Blakeley & Iversen 1978). The present study does not show whether the uptake occurs across the luminal or the abluminal side of the microvessel wall or both. An uptake of monoamines into brain microvessel walls has been reported by estimation of formaldehyde-induced fluorescence following incubation of brain DA, NA and their  $\alpha$ -methylated analogues (Hamberger 1967). In the latter study it was found that the uptake was prevented by desipramine, *d*-amphetamine and cocaine to a

similar extent as the uptake into NA-storing nerve terminals of reserpine-pretreated animals.

The efficiency of the  $^3$ H-NA uptake, as reflected by the ratio of radioactivity between the microvessel fraction and the medium, was of the same order as that reported for the uptake into sympathetic nerves under similar conditions (Edvinsson & Öman 1977; Alm et al. 1979) but considerably higher than the extraneuronal uptake in smooth musculature (Alm et al. 1979). The energy-dependency of the microvessel uptake was confirmed by the inhibition accomplished by hypothermia, combined anoxia and glucose deprivation or uncoupling of the oxidative phosphorylation with dinitrophenol. The results from the incubations with low sodium and high potassium, as well as in the presence of ouabain, suggest that the uptake is linked with a Na/K-dependent ATPase. The uptake was inhibited both with compounds that are known to affect the neuronal (cocaine, desipramine, phenoxybenzamine) and extraneuronal (normetanephrine, oestradiol, phenoxybenzamine) transport of monoamines. This shows that, although the accumulation in brain microvessels is extraneuronal, it has several features in common with the axonal uptake process, however without the subsequent cytoplasmic retention of the amine by a reuptake-sensitive storage mechanism (Bertler et al. 1966). Thus, the uptake and accumulation of amines in the cerebral microvessels in many respects resembles the uptake shown to occur in the wall of pulmonary microvessels (for review, see Gillis 1976) though a difference in the degree of uptake between the various amines could not be established.

The extraction of trace-amounts of neurotransmitter monoamines from the brain circulation during a single capillary passage is only about 3–5% (Oldendorf 1971; Hardebo et al. 1977; Hardebo & Nilsson 1979). Only at high circulating concentrations and after inhibition of the MAO activity is it possible by fluorescence microscopy to visualize a weak accumulation of monoamines in the brain microvessel wall (Hardebo et al. 1979b). The poor penetrability across the luminal endothelial membrane is due to the unity of the structural blood-brain barrier (Reese & Karnovsky 1967) which to a large extent impedes the passage of these water-soluble and polar substances. On the other hand, when the blood-brain barrier is opened experimentally, an uptake and accumulation of monoamines in

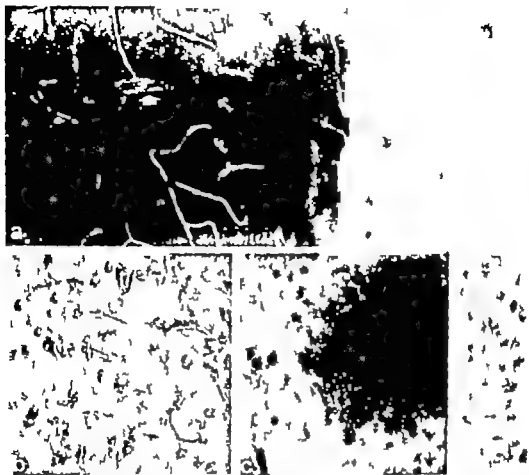


Fig. 2 Fluorescence photomicrographs of brain tissue slices following incubation in the presence of catecholamine uptake inhibitors. (a)–(c) Human temporal cortex ( $\times 90$ ) (a) Intense fluorescent fluorescence in the walls of microvessels but no accumulation in arterioles (arrow) after incubation with  $10 \mu\text{g/ml}$  nortriptyline. (b) In the presence of ouabain  $10^{-4} \text{ M}$  the degree of noradrenaline accumulation is markedly reduced. (c) During hypoxia only very little noradrenaline accumulates in the microvessel wall. (d)–(e) Rat parietal cortex. (d) Intense fluorescence in the walls of capillaries and venules (x) but not in arterioles (arrow) after incubation with  $10 \mu\text{g/ml}$  desipramine. (e) Desipramine ( $10^{-4} \text{ M}$ ) reduces the microvascular accumulation so that only the more voluminous structures are clearly visible.  $\times 75$ .

slices pretreated with nialamide were incubated in the presence of increasing concentrations of NA. A maximum fluorescence was seen at the  $70 \mu\text{g/ml}$  concentration. It has been shown in model experiments that there is a linear relationship between fluorescence intensity and the catecholamine concentration. Due to quenching of the fluorescence at high catecholamine concentrations a further increase in the concentration above a certain level gives an unchanged or even slightly diminished intensity (Ritzén 1966, Jonsson 1971). However, our finding most probably reflects an upper limit for accumulation in the microvessel wall rather than being the result of quenching which occurs only at very high local concentrations.

The inhibitory influence on the uptake of various drugs or by modification of the metabolism listed in Fig. 1 and illustrated in Fig. 2. The effect of inhibition was the same for the other amines studied except for 5-HT which was resistant to desipramine treatment.

**Incubation of microvessels.** An uptake and accumulation of radioactivity was obtained in the microvessel fraction after incubation in the presence of tritiated NA as evidenced by a several higher radioactivity than in the incubation medium (Fig. 3). The uptake was significantly reduced by blockade of  $\text{Na}^+/\text{K}^+$ -dependent ATPase with ouabain by incubation under anaerobic conditions or in the absence of glucose or  $\text{Ca}^{2+}$  (Fig. 3).

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the cells of the microvessel wall is clearly distinguishable (Flodmark et al 1969; Hardebo et al 1979a). Under these conditions the monoamines may enter the cytoplasm of the endothelial cells by pinocytosis (Hansson, Johansson & Blomstrand 1975) or it may pass between (or through) the endothelial cells to reach the abluminal side of the endothelial membrane and from here enter the endothelial cell as well as the pericyte. It can be assumed that a sufficient inactivating mechanism for extraneuronal monoamines in the brain parenchyma is of fundamental value for maintaining an adequate brain function: a clearance process via the brain microvessel walls may work as a local complement to the neuronal re-uptake mechanism and enzymatic breakdown. It is therefore not unexpected that the active uptake of monoamines into the microvessel walls is almost exclusively working across the abluminal membrane of the endothelial cell in direction from the brain into the cytoplasm of this cell (and of the pericyte). This would explain why monoamines following intraparenchymal (Bertler et al 1966) or intraventricular infusion (Fuxe & Ungerstedt 1968 and own unpublished observations) accumulate in the microvessel walls in a narrow zone around the stich channel and periventricularly respectively.

Pre-treatment with the MAO inhibitor nialamide enhanced the *in vitro* accumulation of monoamine into the endothelial cells and pericytes of the microvessel wall. This finding offers further support for the presence of MAO in these cells (Bertler et al 1966; Spector et al 1977; Hardebo et al 1977 and 1979b).

The study has shown the presence of a saturable energy-dependent transport of monoamines into brain microvessel walls. The process shares characteristics with both neuronal and extraneuronal monoamine uptake mechanisms and resembles the microvascular transport of NA and 5-HT in the lung. Once entering the microvessel wall the monoamines are effectively metabolized by the locally present MAO. Although monoamines penetrate the luminal membrane of the cerebral microvessel only to a minor extent, MAO will provide an inactivation of those who do enter. However, the major inactivation mechanism at the level of the microvessel is accomplished by the transport and metabolism of amines in the direction from the brain parenchyma. Both aspects of this inactivation at the blood-brain interphase may assist in securing

an adequately low level of neurotransmitter in the extracellular compartments. However, in certain pathological conditions—marked impairment of morphologic barrier capacity and brain anoxia—this inactivation mechanism is inefficient. The transient increase in neuronal monoamine levels that occurs in the extravascular fluid compartment initially during brain anoxia (Meyer et al 1973, 1974; see also Wenzel & Zervas 1974)—that may be detrimental to the brain by increasing the metabolism in the chemico-anoxic brain area—may in part be due to impaired uptake of the transmitter into the microvascular wall locally.

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# Sulphonylurea (Glibenclamide) enhances somatostatin inhibits glucagon release induced arginine

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K. Sulphonylurea (glibenclamide) enhances somatostatin and inhibits glucagon release  
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ISSN 0001-6772. Department of Endocrinology, Karolinska Hospital, Department of  
Pharmacology, Karolinska Institute, Stockholm, Sweden and Hoechst Aktiengesellschaft,  
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Arginine significantly stimulated the release of insulin, glucagon and somatostatin from the  
isolated perfused rat pancreas. A sulphonylurea, glibenclamide, markedly enhanced the  
effect of arginine on somatostatin release and inhibited its effect on glucagon release.  
Insulin release was not modulated by addition of glibenclamide. These findings support the  
idea of a paracrine interaction of islet hormones.

**Key words:** Somatostatin, glucagon, insulin, perfused rat pancreas.

It has been generally accepted that the principle  
of action of the sulphonylureas is through  
stimulation of insulin release from the pancreatic  
islets (Loubatieres 1946, Grodsky et al 1967, Cerasi  
1969, Loubatieres et al 1970, Malaisse et al  
1978). Recently this opinion was seriously ques-  
tioned, and it was suggested that the chronic an-  
tidiabetic action of the sulphonylureas could be  
related to one or several extrapancreatic effects  
of the drug (Lebovitz et al 1977, Feinglos & Lebo-  
vitz 1978). Moreover, it has been demonstrated that  
sulphonylureas administered *in vivo* to humans and  
monkeys increase the number of insulin receptors and  
strongly enhance insulin sensitivity (Feinglos  
& Lebovitz 1978, Sorensen 1978). In addition,  
several authors report that sulphonylureas suppress  
glucagon release which could explain, at least partly,  
the antidiabetic action of these drugs (Lebovitz  
et al 1969, Loubatieres et al 1970). The latter  
finding could not be confirmed by other investi-  
gators (Peri et al 1977, Baummeister et al 1978).  
The present study demonstrates that one sul-

phonylurea, glibenclamide, suppressed arginine in-  
duced glucagon release which was most likely the  
result of enhanced release of somatostatin—the  
third pancreatic hormone.

## MATERIAL AND METHODS

The experiments were performed on isolated perfused rat  
pancreas. The perfusate Krebs-Ringer bicarbonate so-  
lution to which was added 0.8 g/l of glucose and 20 g/l of  
bovine albumin was administered into the coeliac artery  
and ran into the prepared pancreas by an open circuit.  
The flow-rate of the perfusate was 2.5 ml/min. Insulin was  
determined by double-antibody radioimmunoassay  
(Hales & Randle 1963) using insulin respekt kit  
(Radiochemical centre, Amersham) and a rat insulin stan-  
dard. Glucagon was assayed by the charcoal separation  
technique using 30K antibodies (Agricola Parada et al  
1969). Somatostatin was measured by a radioimmunoas-  
say using own antibodies (K 141E) (Efendić et al 1978).  
The sensitivity of this assay was about 5 pg/ml. Cross-  
reactivity of the antibody was less than 0.01% with insulin,  
glucagon substances P, LH-RH, vasopressin and  
oxytocin. The antigenic specificity of the antibodies was  
determined using somatostatin analogues (Aricourt et al  
1978).



enhancement of somatostatin release. This is a paracrine action of somatostatin. Such a hypothesis calls for an explanation of the absence of insulin release in our experiments. At present, we can visualize two alternatives. First, that glibenclamide enhances arginine-induced insulin release and that this is then counteracted by somatostatin, the net effect being undetectable arginine-induced insulin release. Secondly, in the rat pancreas, the somatostatin-producing cells are closely connected with the glucagon-secreting A-cells but not with the B-cells, the net action of somatostatin would predominantly be exerted on the A-cells. The latter alternative is supported by the finding that addition of antiserum to rat islets markedly stimulates glucagon but not insulin release (Barden et al. 1974). The present findings suggest that inhibition of glucagon release may be one of the modes of action of sulphonylureas and that somatostatin may be the mediator of this inhibition. The significance of such an action in human diabetes remains to be studied. Nevertheless, our findings in the rat system used may open a new approach in the search for oral antidiabetic drugs.

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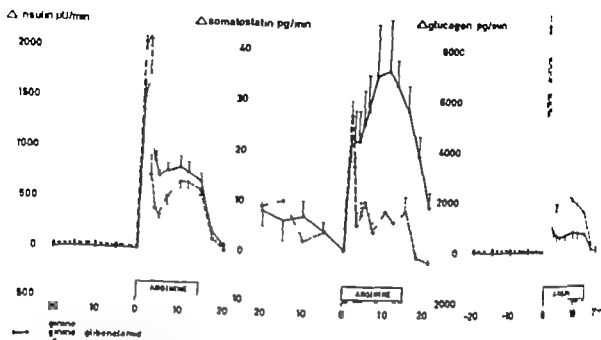


Fig. 1 Effect of arginine in the absence (○—○) and presence (●—●) of glibenclamide on the release of insulin, somatostatin and glucagon from the isolated perfused rat pancreas. The pancreases were equilibrated for 30 min before administration of arginine and glibenclamide. The values are Means  $\pm$  S.E. of eight parallel experiments.

## RESULTS

Administration of arginine (5 mg/ml) significantly enhanced insulin, glucagon and somatostatin release from the pancreas (Table 1). The release pattern was biphasic for all three hormones (Fig. 1). Addition of glibenclamide (1  $\mu$ g/ml) significantly enhanced the effect of arginine on somatostatin re-

lease but inhibited its effect on glucagon release. Insulin release was not significantly altered.

## DISCUSSION

We believe that the inhibition of arginine-induced glucagon release by glibenclamide can be ex-

Table 1 Effect of Glibenclamide on arginine induced release of insulin, glucagon and somatostatin from isolated perfused rat pancreas (mean  $\pm$  S.E.,  $n=8$ )

Response*	Arginine	Arginine + glibenclamide	P
Insulin			
0-6 min	6701 $\pm$ 496	6947 $\pm$ 389	NS
7-15 min	4919 $\pm$ 494	6117 $\pm$ 700	NS
0-15 min	11121 $\pm$ 980	13065 $\pm$ 974	NS
Glucagon			
0-6 min	70166 $\pm$ 966	10709 $\pm$ 1797	<0.01
7-15 min	15864 $\pm$ 683	5967 $\pm$ 1097	<0.005
0-15 min	36070 $\pm$ 9629	16176 $\pm$ 2315	<0.01
Somatostatin			
0-6 min	76 $\pm$ 14	170 $\pm$ 47	NS
7-15 min	5 $\pm$ 17	767 $\pm$ 5	<0.005
0-15 min	18 $\pm$ 79	416 $\pm$ 9	<0.01

\* Integrated responses for insulin in  $\mu$ U  $\times$  min  $\times$  ml for glucagon and somatostatin in pg  $\times$  ml.

# Adrenomedullary control of muscular and hepatic glycogenolysis and of pancreatic hormonal secretion in exercising rats

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We have previously shown that in exercising adrenodemedullated rats chemically sympathectomized with 6-hydroxydopamine, the muscular and hepatic glycogen breakdown were diminished and the plasma glucagon and insulin concentrations were lower and higher respectively than in controls. To elucidate whether these effects could be ascribed selectively to the lack of the adrenal medulla or to the lack of the sympathetic nerve endings, rat were either surgically adrenodemedullated, chemically sympathectomized with 6-hydroxydopamine both adrenodemedullated and chemically sympathectomized or sham-treated. 3 weeks after adrenodemedullation liver biopsy and cardiac blood was obtained. Subsequently the rats either rested or swam with a tail weight for 100 min. Immediately afterwards, cardiac blood was drawn and liver and muscle tissue were collected. The effects of combined adrenodemedullation and chemical sympathectomy were found to be due to the lack of the adrenal medulla. Furthermore, in adrenodemedullated rats, compared to controls the concentration of noradrenaline in plasma was markedly reduced during exercise. The total activity ( $\alpha + \beta$ ) of glycogen phosphorylase in muscle was reduced 20% by adrenodemedullation. The findings indicate that during prolonged exercise adrenodemedullary hormones enhance muscular and hepatic glycogenolysis as well as glucagon secretion but inhibit insulin secretion in the rat. Furthermore, in contrast to the findings in man, in the rat major part of the circulating noradrenaline is of adrenodemedullary origin during exercise.

**Key words:** Exercise, noradrenaline, noradrenaline, sympathetic nervous system, muscles, liver, glycogen, glucagon, insulin, glycogen phosphorylase.

We have previously shown (Galbo et al. 1978) that adrenodemedullation combined with chemical sympathectomy alters the metabolic and hormonal responses to exercise in rats. Thus muscular and hepatic glycogen breakdown were diminished and the plasma concentrations of glucagon and insulin were lower and higher respectively compared to control rats. These findings raised the question as to what extent the differences between adrenodemedullated and chemically sympathectomized rats versus control rats were due to either the lack of the adrenal medulla or to the lack of the peripheral sympathetic nerve endings. Further

more adrenodemedullation inevitably damages some of the adrenal cortex. Accordingly the effects of adrenodemedullation might to some extent be due to decreased cortical function. Finally the sympatho-adrenal system might be of importance for the turnover of muscular enzymes. Thus the effect of adrenodemedullation and/or chemical sympathectomy might be exerted through a decrease in glycogenolytic enzyme concentrations in muscle since these procedures were carried out two weeks before the acute exercise test.

In the present study we have tried to elucidate to what extent the effects of combined adre-



1) The effect of exercise, adrenomedullation and 6-hydroxydopamine on hepatic and muscular glycogen concentrations (expressed as  $\mu\text{mol/g wet weight}$ ) and on concentrations of lactate ( $\text{mmol/l}$ ) and glucose ( $\text{mmol/l}$ ) in blood

are mean  $\pm$  S.E. The number of observations is shown in parenthesis. Rats were exercised (exercise) or resting (rest), adrenomedullated (DM) or sham-operated (DSM) and treated with 6-hydroxydopamine (6-OHD<sup>+</sup>) or not treated (6-OHD<sup>-</sup>). The hepatic glycogen concentration and the blood glucose concentration were measured both before and after rest and exercise in every rat, whereas the remaining variables were measured only after exercise or after rest. The concentrations of both hepatic glycogen and of glucose in blood before exercise or after rest were similar in all groups of rats, and averaged ( $\text{X} \pm \text{S.E.}$ )  $415 \pm 8$  ( $n=66$ ) and  $5.76 \pm 0.06$ ,  $n=66$ , respectively. For each variable all mean values differ significantly from each other ( $p < 0.05$ ) except for mean values obtained by 6-OHD<sup>-</sup> in DM and DSM groups and/or in 6-OHD<sup>-</sup> and 6-OHD<sup>+</sup> groups did not differ significantly and were pooled.

	Exercise		Exercise	
	DM	DSM	DM	DSM
glycogen in hepatic glycogen				
6-OHD <sup>+</sup>	164 $\pm$ 14	41 $\pm$ 14	85 $\pm$ 10	
6-OHD <sup>-</sup>	(17)	(16)	(33)	
glycogen in superficial muscle				
6-OHD <sup>+</sup>	42 $\pm$ 1	73 $\pm$ 2	46 $\pm$ 1	35 $\pm$ 1
6-OHD <sup>-</sup>	(15)	(8)	(16)	(17)
		28 $\pm$ 2		
		(9)		
glycogen in deep ventral muscle				
6-OHD <sup>+</sup>	38 $\pm$ 1	19 $\pm$ 1	46 $\pm$ 1	35 $\pm$ 1
6-OHD <sup>-</sup>	(17)	(17)	(16)	(17)
glycogen in soleus muscle				
6-OHD <sup>+</sup>	43 $\pm$ 1	18 $\pm$ 1	44 $\pm$ 1*	29 $\pm$ 2
6-OHD <sup>-</sup>	(17)	(17)	(16)	(17)
glucose in blood				
6-OHD <sup>+</sup>	2.81 $\pm$ 0.42	6.67 $\pm$ 0.41	1.57 $\pm$ 0.30	
6-OHD <sup>-</sup>	(16)	(17)	(31)	
lactate in blood glucose				
6-OHD <sup>+</sup>	0.72 $\pm$ 0.32*		0.12 $\pm$ 0.28*	
6-OHD <sup>-</sup>	(25)	3.75 $\pm$ 0.53	(33)	
		(9)		

\* values were pooled. Therefore if nothing else is said, all the numbers in the tables for each variable are significantly different ( $p < 0.05$ ) from each other.

## RESULTS

In control rats (sham-operated and sham-injected) glycogen concentration in skeletal muscle had significantly decreased after 100 min of swimming. The decrease in the hepatic glycogen concentration was during exercise significantly larger than during the equivalent period of rest (Table 1). During exercise in these rats the concentrations in plasma of glucagon and cortisol and of glucose and lactate in blood increased (Tables 1, 3 and 4), whereas the concentration of insulin in plasma did not change significantly (Table 2). No consistent effects of exercise, adrenomedullation or 6-by-

droxydopamine on the hematocrit or on the concentrations of FFA and glycerol in serum were found (not shown). Body weight was neither influenced by adrenomedullation nor by chemical sympathectomy. The mean was  $270 \pm 2$  g ( $\text{X} \pm \text{S.E.}$ ,  $n=67$ ).

### Effects of adrenomedullation

In resting rats adrenomedullation increased the concentrations of muscular glycogen (Table 1) and plasma insulin (Table 3), and decreased the concentration of glucagon in plasma (Table 3). During exercise the decreases in muscular and hepatic glycogen concentrations were markedly reduced by adrenomedullation (Table 1). Furthermore, after exercise the concentrations of glucose and lactate in blood and of glucagon in plasma were lower and the concentration of insulin in plasma was higher in

nodemedullation and chemical sympathectomy could be ascribed to the lack of the adrenal medulla or to the lack of the peripheral sympathetic nerve endings. Rats were either demedullated, chemically sympathectomized, both demedullated and sympathectomized or sham treated and were either resting or exercised by swimming. The application of an *in vivo* liver biopsy technique made it possible more accurately to determine the exercise induced hepatic glycogen breakdown. The enzyme glycogen phosphorylase is of importance for muscular glycogen breakdown capacity. Accordingly we measured the total activity ( $a+b$ ) of phosphorylase in muscle. Finally the plasma concentration of cortisol was measured as an indicator of adrenocortical function.

## MATERIALS AND METHODS

67 male Wistar rats weighing 180–200 g were anesthetized with ether and either adrenodemedullated or sham-operated. Adrenodemedullation was performed by electrocoagulation after lumbar incisions. No supplement of saline was given after demedullation. In order to allow regeneration of the adrenal cortex the surgery was carried out 21 days prior to the exercise test.

In half of both the adrenodemedullated and the sham-operated rats chemical sympathectomy was accomplished. The procedure which has been found to result in the most complete destruction of adrenergic nerve terminals in rats (Thoenen & Tranzer 1968) was used. Accordingly one week after the surgery each rat received two injections of 34 mg/kg b.w. 6-hydroxydopamine dissolved immediately before injection in 0.5 ml 0.9% NaCl containing 0.5 mg ascorbic acid. The two injections were given in a tail vein 6 h apart. One week later the rats received further 2 × 68 mg/kg b.w. 6-hydroxydopamine *i.v.* In the other half of the rats only the solvent was injected.

The exercise test was carried out in the morning and the rats fasted 1–2 h in advance. During ether anesthesia (0.5 ml of blood were drawn by cardiac puncture for glucose and hematocrit analysis. Then a small biopsy (15–40 mg) of the liver was obtained through a minute abdominal incision which afterwards was sutured. Bleeding was negligible. After 15 min of recovery the rats either swam 100 min with a tail weight of lead (7% of body weight) in water maintained at 33–34°C or they remained resting. At the end of exercise or the equivalent period of rest the rat were quickly anesthetized with ether and 8 ml of blood were drawn by cardiac puncture. Then samples of the liver of the superficial part of the vastus lateralis muscle (which consists predominantly of fast-twitch white fibers) of the deep part of the vastus lateralis muscle (predominantly fast-twitch red fibers) and of the soleus muscle (predominantly slow-twitch red fibers) were quickly removed and frozen in liquid nitrogen. Blood and tissue sampling was completed within 5 min after exercise. In

adrenodemedullated rats the selective destruction of adrenal medulla was verified by light microscopy of hematoxylin and eosin stained sections of each gland.

The methods of analysis of blood and tissue lactates, hematocrit, glucagon and catecholamines have been reported elsewhere (Galbo & Høj 1974a; Galbo 1977a). Insulin was determined by radioassay using rat insulin as the standard. Within assay coefficient of variation ( $n=70$ ) was 3%. At a plasma insulin concentration (rat plasma) of 400 pmol/l recovery of added insulin in final concentrations between 400 and 1000 pmol/l yielded values within  $\pm 5\%$  of expected value. Cortisol in plasma was determined by a commercially available solid phase radioimmunoassay kit (Chillick & Lincoln). Plasma from adrenalecctomized rats yields results which were not significantly different from intact ( $a+b$ ) phosphorylase activity in muscle was determined fluorometrically after incubation of the rat homogenate with purified rabbit muscle phosphorylase kinase and ATP (Lowry & Passonneau 1972). The activity was determined as formation of glucose 1-phosphate from glycogen added in excess, and was expressed as  $\mu\text{mol}$  of glucose 1-phosphate/g wet muscle. As a decrease in glycogen concentrations between two samples taken before and after blood drawing might be underestimated due to differences in the blood content of liver. However the difference between the glycogen content in biopsies taken before and immediately after exsanguination in 6 rats was  $1 \pm 5$  ( $\pm 5\text{ E}$ )  $\mu\text{mol}$  of glucose/kg wet weight.

The concentration of glycogen in muscle was measured in wet tissue. Thus changes in water content might influence the measured differences in glycogen concentrations. Therefore the water content was determined in biopsies of the deep part of the vastus lateralis muscle taken from 5 rats from each of the eight groups. Ten samples were weighed before and after freeze drying. No significant differences in water content between the groups were found.

The major part of the data was analyzed by a three-way analysis of variance preceded by Bartlett's test for homogeneity of variance (Snedecor & Cochran 1969). No major discrepancies were found with Bartlett's test. The three treatments (ways) are exercise, adrenal demedullation and injection of 6-hydroxydopamine. Each treatment has two levels. Accordingly exercise and exercise denote that the rats were exercised and resting, respectively. DM and DM denote adrenodemedullated and sham-operation respectively and 6-OHD and 6-OHD denote injection with 6-hydroxydopamine and sham-injections respectively. The plasma concentrations of norepinephrine, epinephrine and cortisol are not measured in all groups of rats. These data were analysed by a two-way analysis of variance (Snedecor & Cochran 1965). By the analysis of variance it is calculated, and to what extent a certain treatment or combination of treatments (interactions) influence the data. If a certain treatment or combination does not influence the data significantly the data can be pooled paying no attention to the treatment or combination of treatments. Accordingly for each variable mean  $\pm$  S.E. is calculated. If it did not

### 3) The effect of exercise adrenomedullary 6-hydroxydopamine on the concentration of norepinephrine in plasma (pmol l<sup>-1</sup>)

were exercised (exercise) or resting (exercise), adrenomedullated (DM<sup>+</sup>) or sham-operated (DM<sup>-</sup>) and infused with 6-hydroxydopamine (6-OHD<sup>+</sup>) or sham-infused (6-OHD<sup>-</sup>). No interactions between the three effects were found. This fact allows data to be presented as one total mean and an estimated value for each effect.

mean of treatments	56±3
exercise	-22±3
DM <sup>+</sup>	17±3
6-OHD <sup>+</sup>	+9±3
exercise	22±3
DM <sup>-</sup>	-17±3
6-OHD <sup>-</sup>	-9±3

are mean±S.E. The estimated mean of any is found by adding the appropriate estimated effects total mean. The mean concentration in the group exercise 6-OHD<sup>+</sup> is 56-22+17.9=42±6. The on the result is  $\sqrt{4^2+3^2}$ .

abolic and hormonal responses to exercise was the destruction of the peripheral sympathetic endings by chemical sympathectomy only slight effects. Thus in adrenomedullated the exercise-induced muscular and hepatic glycogen breakdowns were markedly reduced and plasma concentrations of glucagon and insulin were lower and higher respectively compared to findings in control rats (Tables 1, 2 and 3). In line with the reduced exercise-induced muscular and hepatic glycogenolysis were the findings of lower post-exercise blood lactate and glucose concentrations in demedullated rats than in controls (Table 1). In adrenomedullated rats circulating norepinephrine was after exercise undetectable in 10 of 13 rats and the plasma norepinephrine concentration was markedly reduced (Table 3). These data indicate both that the procedure of adrenomedullation was efficient and that, in contrast to the findings in man (Vendtsen 1960) in the rat a major part of the circulating norepinephrine is of adrenomedullary origin during exercise. We have previously shown (Galbo et al. 1978) that the concentrations of norepinephrine in muscle, pancreas and liver are reduced to 27%, 12% and 5% respectively of control values by chemical sympathectomy.

Other studies have demonstrated an only minor

influence (Struck & Tipton 1974; Sembrowich et al. 1974) or no influence at all (Gollnick et al. 1970; Malling et al. 1966) of adrenomedullation on glycogenolysis in exercising rats. The difference between these observations and the findings in the present study could possibly be due to differences in the state of training of the animals or in the applied exercise procedures or intensities. Furthermore in the present study the application of a liver biopsy technique made it possible to determine the exercise-induced hepatic glycogen breakdown in individual rats. Accordingly we were probably able to measure hepatic glycogen breakdown more accurately than in other studies. In accordance with our results administration of  $\beta$ -receptor blocking agents to rats has been shown to decrease the hepatic glycogen breakdown during swimming (Kindler et al. 1978). Furthermore infusion of the  $\beta$ -adrenergic blocking agent propranolol to steadily exercising dogs decreased the rate of muscular glycogen breakdown and was accompanied by a marked fall in the plasma lactate concentration and by a more rapid decrease in the plasma glucose concentration in comparison with control experiments (Issekutz 1978). In keeping with these findings  $\beta$ -adrenergic blockade has been shown to diminish the reduction in glycogen content in an in situ isolated, stimulated muscle preparation (Costin et al. 1971).

In rats with an intact adrenal medulla chemical sympathectomy increased the exercise-induced muscular glycogenolysis in the fast-twitch muscle fibers (Table 1). This finding might be due to impaired cardiovascular adjustments to exercise lead-

Table 4. The effect of exercise and adrenomedullation on the concentration of cortisol in plasma (nmol l<sup>-1</sup>)

Values are mean±S.E. The number of observations is shown in parentheses. Rats were either exercised (exercise<sup>+</sup>) or resting (exercise<sup>-</sup>) and adrenomedullated (DM<sup>+</sup>) or sham-operated (DM<sup>-</sup>). The mean values differ significantly ( $p<0.05$ ) from each other. Exercise values did not differ significantly and were therefore pooled.

	DM <sup>+</sup>	DM <sup>-</sup>
Exercise <sup>+</sup>	199±32 (7)	309±30 (8)
Exercise <sup>-</sup>	119±21 (16)	

Table 2 The effect of exercise, adrenodemedullation and 6-hydroxydopamine on the plasma concentrations of insulin ( $\text{pmol l}^{-1}$ ), norepinephrine ( $\text{nmol l}^{-1}$ ) and epinephrine ( $\text{nmol l}^{-1}$ ) and on the activity ( $a+b$ ) of glycogen phosphorylase in muscle (expressed as  $\mu\text{mol}$  of glucose 1 phosphate released  $\text{min}^{-1}$ )

Values are mean  $\pm$  S.E. The number of observations is shown in parentheses. Rats were exercised (exercise) or not exercised (DM), adrenodemedullated (DM) or sham-operated (DM) and treated with 6-hydroxydopamine (6-OHD) or sham-treated (6-OHD<sup>-</sup>). For each variable the mean values differ significantly ( $p < 0.05$ ) from each other if indicated by exercise and exercise groups and/or in 6-OHD and 6-OHD<sup>-</sup> groups did not differ significantly they were pooled.

	DM		DM	
	Exercise	Exercise	Exercise	Exercise
Insulin in plasma				
6-OHD				
6-OHD		656 $\pm$ 34 (33)		210 $\pm$ 34 (33)
Norepinephrine in plasma				
6-OHD				
6-OHD	6.2 $\pm$ 2.3 (13)	Not measured	19.3 $\pm$ 1.1 (14)	Not measured
Epinephrine in plasma				
6-OHD				
6-OHD	Undetectable in 10 out of 13 rats	Not measured	11.9 $\pm$ 1.1 (14)	Not measured
Phosphorylase ( $a+b$ ) activity in muscle				
6-OHD				
6-OHD	11.3 $\pm$ 0.5 (26)		14.2 $\pm$ 0.5 (9)	

adrenodemedullated rats than in controls (Tables 1, 2 and 3).

The concentrations of norepinephrine and epinephrine were measured after exercise only. Adrenodemedullation decreased the plasma concentration of epinephrine to below detection limit (less than 0.01  $\text{pmol}$  in 50  $\mu\text{l}$  plasma = 0.2  $\text{nmol l}^{-1}$ ) in 10 out of 13 rats (Table 2). The three detectable values were 0.2, 0.3 and 0.4  $\text{nmol l}^{-1}$ . Furthermore, demedullation reduced the norepinephrine concentration in plasma markedly (Table 2).

The plasma concentration of cortisol was at rest not significantly different in demedullated and in control rats (Table 4). After exercise an increase in the concentration of cortisol was found in both demedullated and in control rats. The increase however was largest in control rats (Table 4).

The activity of total ( $a+b$ ) glycogen phosphorylase in muscle was decreased 70% by adrenodemedullation (Table 2).

#### Effects of 6-hydroxydopamine (6-OHD)

The combination of adrenodemedullation and treatment with 6-hydroxydopamine did not produce more marked effects than did adrenodemedullation alone (Tables 1, 2 and 3). In rats with an intact adrenal medulla, treatment with 6-OHD increased

the exercise induced glycogen breakdown in the superficial part of the vastus lateralis muscle compared to the breakdown in control rats (Table 2). A similar tendency was found in the deep part of the vastus lateralis muscle (not shown). Furthermore, destruction of the peripheral sympathetic nerve endings by 6-OHD in rats with an intact adrenal medulla abolished the exercise induced increase in blood glucose concentration (Table 1). In both adrenodemedullated rats and in rats with an intact adrenal medulla 6-OHD increased the concentration of glucagon in plasma at rest and after exercise (Table 3).

Treatment with 6-OHD did not significantly reduce the plasma concentration of norepinephrine (Table 2). However, in rats with an intact adrenal medulla the ratio norepinephrine/epinephrine was decreased by 6-OHD [ $7.4 \pm 0.5$  vs.  $17 \pm 0.9$  (S.E.  $p < 0.02$ ,  $t$  test)] due to a tendency towards both a lower concentration of norepinephrine (2) and a higher concentration of epinephrine (1) compared to controls.

#### DISCUSSION

The present study has shown that adrenodemedullation has a

in the rat which influences

*The effect of exercise adrenodemedullated 6-hydroxydopamine on the concentration of norepinephrine in plasma (pmol l<sup>-1</sup>)*

are exercised (exercise) or resting (exercise<sup>-</sup>), adrenodemedullated (DM) or sham-operated (DM<sup>+</sup>) and all 6-hydroxydopamine (6-OHD<sup>+</sup>) or sham-operated (6-OHD<sup>-</sup>). No interactions between the three rats were found. This fact allows data to be presented as total mean and an estimated value for each factor.

Mean of treatments	% ± 3
DM	-22 ± 3
DM <sup>+</sup>	+17 ± 3
DM <sup>+</sup>	+9 ± 3
DM <sup>+</sup>	+22 ± 3
DM <sup>+</sup>	-17 ± 3
DM <sup>+</sup>	9 ± 3

are mean ± S.E. The estimated mean of any factor is found by adding the appropriate estimated effects to the total mean. The mean concentration in the group exercise 6-OHD<sup>+</sup> is 56-22+17.9=42±6. The result is  $F_{4,12}=6$ .

and hormonal responses to exercise and the destruction of the peripheral sympathetic endings by chemical sympathectomy only slight effects. Thus in adrenodemedullated rats the exercise-induced muscular and hepatic glycogen breakdown were markedly reduced and plasma concentrations of glucose and insulin lower and higher respectively compared to findings in control rats (Tables 1, 2 and 3). In addition to the reduced exercise-induced muscular and hepatic glycogenolysis were the findings of lower post-exercise blood lactate and glucose concentrations in demedullated rats than in controls (Table 1). In adrenodemedullated rats circulating norepinephrine was after exercise undetectable in 10 of 13 rats and the plasma norepinephrine concentration was markedly reduced (Table 2). These findings indicate both that the procedure of adrenodemedullation was efficient and that in contrast to the findings in man (Vendisalu 1960), in the rat a major part of the circulating norepinephrine is of adrenomedullary origin during exercise. We have previously shown (Gæbo et al. 1978) that the concentrations of norepinephrine in muscle, pancreas and liver are reduced to 77%, 1% and 5% respectively of control values by chemical sympathectomy.

Other studies have demonstrated an only minor

influence (Struck & Tipton 1974; Sembrowich et al. 1974) or no influence at all (Goldnick et al. 1970; Maling et al. 1966) of adrenodemedullation on glycogenolysis in exercising rats. The difference between these observations and the findings in the present study could possibly be due to differences in the state of training of the animals or in the applied exercise procedures or intensities. Furthermore in the present study the application of a liver biopsy technique made it possible to determine the exercise-induced hepatic glycogen breakdown in individual rats. Accordingly we were probably able to measure hepatic glycogen breakdown more accurately than in other studies. In accordance with our results administration of  $\beta$ -receptor blocking agents to rats has been shown to decrease the hepatic glycogen breakdown during swimming (Kindler et al. 1978). Furthermore infusion of the  $\beta$ -adrenergic blocking agent propranolol to steadily exercising dogs decreased the rate of muscular glycogen breakdown and was accompanied by a marked fall in the plasma lactate concentration and by a more rapid decrease in the plasma glucose concentration in comparison with control experiments (Isaacs 1978). In keeping with these findings  $\beta$ -adrenergic blockade has been shown to diminish the reduction in glycogen content in an *in situ* isolated, stimulated muscle preparation (Costin et al. 1971).

In rats with an intact adrenal medulla chemical sympathectomy increased the exercise-induced muscular glycogenolysis in the fast-twitch muscle fibers (Table 1). This finding might be due to impaired cardiovascular adjustments to exercise load-

Table 4. *The effect of exercise and adrenodemedullation on the concentration of cortisol in plasma (nmol l<sup>-1</sup>)*

Values are mean ± S.E. The number of observations is shown in parentheses. Rats were either exercised (exercise) or resting (exercise<sup>-</sup>) and adrenodemedullated (DM) or sham-operated (DM<sup>+</sup>). The mean values differ significantly ( $p < 0.05$ ) from each other. Exercise values did not differ significantly and were therefore pooled.

	DM	DM <sup>+</sup>
Exercise	199 ± 37 (7)	309 ± 30 (8)
Exercise <sup>-</sup>	119 ± 21 (16)	



ing to insufficient muscular perfusion to the tendency towards increased plasma epinephrine concentrations intensifying  $\beta$ -adrenergic stimulation or to impaired mobilization of other substrates in sympathectomized rats compared to controls. If the mobilization of other substrates (e.g. fat) during exercise is impeded by sympathectomy, the muscles probably derive more of the necessary energy from glycogen combustion (Rennie & Holloszy 1977).

Regarding the pancreatic hormonal secretion we found that the plasma insulin concentration was markedly increased by adrenomedullation but uninfluenced by exercise and chemical sympathectomy (Table 2). Possibly the lack of steady state in the short period (less than 2 min) between the termination of exercise and blood drawing prevented the finding of a decreased plasma insulin concentration in exercised rats. However in control rats the unchanged insulin concentration during exercise indicates a suppression of insulin secretion since the blood glucose concentration increased in these rats (Table 1). Such a suppression was found in neither adrenomedullated nor sympathectomized rats since the plasma insulin concentration as well as the blood glucose concentration in these rats were unchanged during exercise (Table 1). Thus also the sympathetic nerves seem to inhibit insulin secretion during exercise. Our findings are in keeping with the increase at rest and after exercise in the plasma insulin concentration found after administration of the  $\alpha$ -receptor blocking agent phentolamine (Luyckx & Lefebvre 1974) and with the epinephrine induced decrease of the insulin secretion (Wright & Malaisse 1968) in resting rats. Destruction of sympathetic nerves by immunosympathectomy did in a previous study not influence plasma insulin and glucose concentrations during exercise (Luyckx et al 1975). However in that study the work intensity and with that probably sympatho-adrenal activity was lower than in the present study and no sign of inhibition of insulin secretion was found in exercising control rats.

As to glucagon, exercise and sympathectomy increased whereas adrenomedullation decreased the plasma glucagon concentration (Table 3). The decrease in the plasma glucagon concentration due to adrenomedullation probably reflects the reduction of  $\beta$ -adrenergic enhancement of glucagon secretion (Gench et al 1976; Luyckx & Lefebvre 1974). The increase in the plasma glucagon con-

centration found after chemical sympathectomy is in contrast to findings describing enhanced glucagon secretion by the sympathetic nervous system (Gench et al 1976; Luyckx et al 1975). Our findings are not easily explained. However the decreased plasma glucagon concentration found during exercise intensity during short term exercise in man (Galbo, personal communication) points to an inhibiting influence of the sympatho-adrenal system upon the amount of glucagon reaching the portal blood stream. During exercise an increase in plasma glucagon concentration which could be ascribed to sympatho-adrenal influence was not found (Table 3). Thus in the rat and more probably in man (Galbo et al 1976b) the glucagon response to exercise cannot entirely be ascribed to stimulation by the sympatho-adrenal system. There has been put forward that during exercise the plasma glucose concentration influences glucagon secretion (Galbo et al 1977b; Luyckx & Lefebvre 1974). This is probably so during prolonged exercise when a decrease in the plasma glucose concentration may enhance glucagon secretion (Harvey et al 1977b; Harvey et al 1974). In the present study however a decline in the blood glucose concentration was not found during exercise. It cannot be excluded that the lack of steady state in the short period after the end of exercise and the blood drawing prevented the demonstration of a decrease in the glucose concentration.

The findings in the present study imply that during prolonged exercise circulating catecholamines originating in the adrenal medulla enhance muscular and hepatic glycogenolysis and glucagon secretion but inhibit insulin secretion. However it has been clarified whether the catecholamines do enhance glycogen breakdown and glucagon secretion during exercise or whether these effects are due to a primary inhibition of insulin secretion. Insulin is able to inhibit glucagon secretion (Luyckx et al 1976) and to promote glycogen storage. Probably the reduced concentration of glucagon in adrenomedullated rats did not play any role in the lower exercise induced hepatic glycogenolysis. This is so since during exercise the action of glucagon on hepatic glycogenolysis seems to be permissive. In exercising dogs and rats the hepatic glucose production may proceed unchanged in the absence of an increase in the plasma glucagon concentration (Vranic et al 1979; Galbo et al 1977b). In the present study the plasma glucagon concentration was decreased if



ing to insufficient muscular perfusion to the tendency towards increased plasma epinephrine concentrations intensifying  $\beta$ -adrenergic stimulation or to impaired mobilization of other substrates in sympathectomized rats compared to controls. If the mobilization of other substrates (e.g. fat) during exercise is impeded by sympathectomy the muscles probably derive more of the necessary energy from glycogen combustion (Rennie & Holloszy 1977).

Regarding the pancreatic hormonal secretion we found that the plasma insulin concentration was markedly increased by adrenomedullation but uninfluenced by exercise and chemical sympathectomy (Table 7). Possibly the lack of steady state in the short period (less than 2 min) between the termination of exercise and blood drawing prevented the finding of a decreased plasma insulin concentration in exercised rats. However in control rats the unchanged insulin concentration during exercise indicates a suppression of insulin secretion since the blood glucose concentration increased in these rats (Table 1). Such a suppression was found in neither adrenomedullated nor sympathectomized rats since the plasma insulin concentration as well as the blood glucose concentration in these rats were unchanged during exercise (Table 1). Thus also the sympathetic nerves seem to inhibit insulin secretion during exercise. Our findings are in keeping with the increase at rest and after exercise in the plasma insulin concentration found after administration of the  $\alpha$ -receptor blocking agent phentolamine (Luyckx & Lefebvre 1974) and with the epinephrine induced decrease of the insulin secretion (Wright & Malaisse 1968) in resting rats. Destruction of sympathetic nerves by immunosympathectomy did in a previous study not influence plasma insulin and glucose concentrations during exercise (Luyckx et al 1975). However in that study the work intensity and with that probably sympatho-adrenal activity was lower than in the present study and no sign of inhibition of insulin secretion was found in exercising control rats.

As to glucagon exercise and sympathectomy increased whereas adrenomedullation decreased the plasma glucagon concentration (Table 3). The decrease in the plasma glucagon concentration due to adrenomedullation probably reflects the reduction of  $\beta$  adrenergic enhancement of glucagon secretion (Gerich et al 1976; Luyckx & Lefebvre 1974). The increase in the plasma glucagon con-

centration found after chemical sympathectomy is in contrast to findings describing enhancement of glucagon secretion by the sympathetic nerve (Gerich et al 1976; Luyckx et al 1979). Our finding is not easily explained. However the decrease in plasma glucagon concentration found with increasing exercise intensity during short term exercise in man (Galbo personal communication) points to an inhibiting influence of the sympatho-adrenal system upon the amount of glucagon reaching the peripheral blood stream. During exercise an increase in plasma glucagon concentration which could not be ascribed to sympatho-adrenal influence was found (Table 3). Thus in the rat and more pronounced in man (Galbo et al 1976b) the glucagon response to exercise cannot entirely be ascribed to enhancement by the sympatho-adrenal system. The concept has been put forward that during exercise the plasma glucose concentration influences glucagon secretion (Galbo et al 1977b; Luyckx & Lefebvre 1974). This is probably so during prolonged exercise when a decrease in the plasma glucose concentration may enhance glucagon secretion (Galbo et al 1977b; Harvey et al 1974). In the present study however a decline in the blood glucose concentration was not found during exercise. But cannot be excluded that the lack of steady state in the short period after the end of exercise and blood drawing prevented the demonstration of a decrease in the glucose concentration.

The findings in the present study imply that during prolonged exercise circulating catecholamines originating in the adrenal medulla enhance muscular and hepatic glycogenolysis and glucagon secretion but inhibit insulin secretion. However it has to be clarified whether the catecholamines directly enhance glycogen breakdown and glucagon secretion during exercise or whether these effects are due to a primary inhibition of insulin secretion. If insulin is able to inhibit glucagon secretion (Clarke et al 1976) and to promote glycogen storage, probably the reduced concentration of glucagon in adrenomedullated rats did not play any role for the lower exercise induced hepatic glycogenolysis. This is so since during exercise the action of glucagon on hepatic glycogenolysis seems to be permissive. In exercising dogs and rats the hepatic glucose production may proceed unchanged in the absence of an increase in the plasma glucagon concentration (Vranic et al 1979; Galbo et al 1979) but is decreased if the plasma glucagon concentration

# Alterations in membrane electrical properties during long-term denervation of rat skeletal muscle

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Some membrane electrical properties of the extensor digitorum longus muscle of the rat were examined up to 21 days after denervation. The resting potential was significantly more depolarized at 3 days after denervation than it was at later times. The rate of rise ( $dV/dt$ ) of the action potential decreased throughout the time course of the study but approached steady value between 14 and 21 days after denervation. In addition, the  $dV/dt$  of tetrodotoxin (TTX)-resistant action potentials increased up to and including 7 days after nerve section, but declined thereafter. When expressed as % of control, the dose-response to TTX was similar throughout denervation. It is suggested that the large depolarization observed early in denervation may be related to the turnover of membrane constituents occurring at this time. The results also suggest that denervation produces a reduction in the number of fast TTX-sensitive Na<sup>+</sup> channels with the appearance of a new population of slow TTX-resistant channels. However, long-term denervation results in a reduction in the density of both types of channels.

**Key words:** Denervation, membrane electrical properties, sarcoplasm, skeletal muscle

It is well known that denervation of mammalian skeletal muscle produces profound changes in its membrane electrical properties. Among these alterations are: a depolarization of the resting potential, a decrease in the rate of rise ( $dV/dt$ ) of the action potential and the appearance of tetrodotoxin (TTX)-resistant action potentials (Albuquerque & Thiesleff 1968; Redfern & Thiesleff 1971*a*, *b*). The impetus for the present study came from the observation that after an initial increase in the  $dV/dt$  of TTX-resistant action potentials early in denervation, the toxin-resistance period to decrease between 5 and 12 days after denervation (Redfern & Thiesleff 1971*b*). The possibility remained that the appearance of TTX-resistant Na<sup>+</sup> channels could be a temporary response that may not persist during long-term denervation. Therefore, the present study was undertaken to examine the effects of long-term denervation on some of the membrane electrical properties of rat skeletal muscle.

## METHODS

All experiments were conducted *in vitro* on the extensor digitorum longus muscle (EDL) of adult male rats (250-

350 g) of the Sprague-Dawley strain. All surgical procedures were done using diethyl ether for anesthesia.

Denervation was performed by removing 3-4 mm section of the deep peroneal nerve about 10 mm from its entrance into the muscle. None of the denervated muscles showed signs of reinnervation during the time course of the study. Control muscles were obtained from contralateral non-denervated limbs, as well as from unoperated rats of equivalent size. At 3, 7, 14 and 21 days after denervation the muscles were excised under continuous flow of oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) Krebs-Ringer solution having the following composition in millimoles per liter: NaCl, 135; KCl, 5; CaCl<sub>2</sub>, 4; MgCl<sub>2</sub>, 1; Na<sub>2</sub>HPO<sub>4</sub>, 1; NaHCO<sub>3</sub>, 15; dextrose, 11. The pH of this solution was 7.2-7.3. The muscles were placed immediately into heated (30±1°C) chamber having a volume of 25 ml and perfused at a rate of 4 ml/min.

After an equilibration period of 20-30 min, recordings of resting membrane potentials and action potentials were made from surface fibers in the extrajunctional region which was defined as the area at least 5 mm from the end-plate zone. For these measurements, glass microelectrodes filled with 3 M KCl and having resistance of 10-15 MΩ were used. Action potentials were generated and recorded directly using two microelectrodes inserted in the same muscle fiber at an interelectrode distance of 50-100 μm. A constant current was passed through one electrode to locally hyperpolarize the muscle fiber to -90 mV. After 10-20 s an action potential was elicited by superimposing rectangular depolarizing pulse of 5 ms duration. Hyperpolarizations greater than -90 mV or

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# Alterations in membrane electrical properties during long term denervation of rat skeletal muscle

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SELLIN V. C. & THESLEFF S. Alterations in membrane electrical properties during long-term denervation of rat skeletal muscle. *Acta Physiol Scand* 1980, 108, 243-46. Received 8 June 1979. ISSN 0001-4772. Department of Pharmacology, University of Lund, Sweden.

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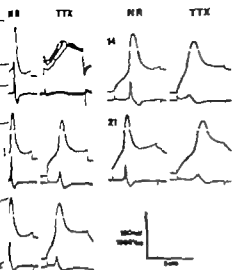


Fig. 1. Action potentials and their first derivatives recorded from the extensor digitorum longus muscle of the rat in Krebs-Ringer solution (NR) and in Krebs-Ringer solution containing  $10^{-6}$  M tetrodotoxin (TTX) at 14, 21 and 28 days after nerve crush (inserted numbers). The action potential indicates the zero potential.

observed previously for the extensor digitorum longus of the rat (Albuquerque & Theleff 1968). However, the resting potential measured here at 3 days after denervation was substantially more depolarized than at later times. This was accompanied by the observation that larger currents were needed to hyperpolarize the membrane at three days after denervation than at other times. This suggests that the applied hyperpolarizing current encountered a reduced 'leak' conductance. It is difficult to determine whether this observation has particular physiological significance. However, it is well known that considerable alterations occur in the membrane at this time, e.g. the appearance of TTX-resistant Na<sup>+</sup> channels and extrajunctional receptors, thus indicating the insertion of new membrane elements. It appears likely that this type of membrane turnover may be explained by the processes of endo- and exocytosis. High rates of endocytosis have been shown to begin at about 2-3 days after denervation (Libelius *et al.* 1978; Libelius, Josefsson & Lundquist, 1979). It is interesting to note that the process of endocytosis in the amoeba is accompanied by both a decrease in resting potential and an increase in membrane conductance (Josefsson, Holmer & Hansson 1975). These observations

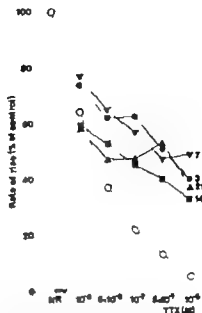


Fig. 2. Effects of various concentrations of tetrodotoxin (TTX) on the rate of rise of action potentials recorded from the extensor digitorum longus (EDL) of the rat. The open circles represent the dose-response curve for the innervated EDL, while the different days after denervation are indicated by the numbers to the right of the curves.

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Tetrodotoxin had no effect on the resting potential of innervated muscles but caused significant hyperpolarization of muscles denervated for 3 and 7 days. A smaller hyperpolarization was demonstrated at 14 days after denervation but none at 21 days. This effect of TTX on denervated muscle was shown previously (Grampp, Harris & Theleff 1972) and may be related to an abnormally high  $P_{Na}$  which has been suggested to occur in the early stages of denervation (Robbins 1977).

The present results show that while the rate of rise ( $dV/dt$ ) of the action potential decreases steadily after denervation, the  $dV/dt$  in the presence of TTX shows a biphasic response to denervation increasing up to 7 days after denervation and decreasing thereafter. Using labelled saxitoxin (STX) Ritchie & Rogart (1977) suggested that the density of STX-sensitive channels remain unchanged after denervation and that toxin resistant action potentials reflect the presence of an entirely new popula-



Table 1 Rate of rise ( $dV/dt$ ) and overshoot of action potentials and resting membrane potential recorded from extensor digitorum longus muscles in normal Krebs Ringer solution (NR) and in Krebs Ringer with  $10^{-6}$  M tetrodotoxin (TTX) at various days after denervation. Each value indicates mean  $\pm$  S.E.

Days after denervation	Fibers/muscles	NR			Fibers/muscles	TTX ( $10^{-6}$ M)		
		$dV/dt$ (V/s)	Overshoot (mV)	Em (mV)		$dV/dt$ (V/s)	Overshoot (mV)	Em (mV)
0	30/7	671 $\pm$ 22	37.8 $\pm$ 1.3	-78.8 $\pm$ 0.6	28/7	-	-	-77.1 $\pm$ 1
3	23/6	536 $\pm$ 29	41.4 $\pm$ 1.7	-51.1 $\pm$ 1.1	34/6	215 $\pm$ 16	26.9 $\pm$ 1.6	-64.4 $\pm$ 1.5
7	36/8	478 $\pm$ 1	40.3 $\pm$ 0.8	-57.2 $\pm$ 1.0	48/8	232 $\pm$ 13	33.3 $\pm$ 1.0	-63.6 $\pm$ 1.5
14	56/12	381 $\pm$ 10	36.8 $\pm$ 0.9	-55.6 $\pm$ 0.8	69/17	124 $\pm$ 9	17.9 $\pm$ 1.3	-69.5 $\pm$ 1.6
21	56/13	331 $\pm$ 9	30.6 $\pm$ 1.0	-56.8 $\pm$ 0.8	61/12	122 $\pm$ 9	17.9 $\pm$ 1.4	-65.9 $\pm$ 1.6

longer than 5 d did not increase appreciably the rate of rise of the action potential. Differentiation of the action potential was accomplished by using an operational amplifier adjusted for maximum negative capacitance. The time constant of the recording system was about 40  $\mu$ s. These procedures were repeated after exposing the muscles to Krebs-Ringer solution containing various concentrations of tetrodotoxin (Sankyo Co. Ltd. Tokyo) for a period of about 20 min.

## RESULTS

Denervation of the extensor digitorum longus (EDL) muscle produced a fall in resting membrane potential from a control value of  $-78.8 \pm 0.6$  mV (Mean  $\pm$  S.E.). This depolarization was greatest at 3 days after denervation ( $-51.1 \pm 1.1$  mV). At 7 ( $-57.2 \pm 0.8$  mV), 14 ( $-55.6 \pm 0.8$  mV) and 21 ( $-56.8 \pm 0.8$  mV) days after denervation the resting potentials were less depolarized and similar in magnitude. When  $10^{-6}$  M tetrodotoxin (TTX) was added to the bathing solution the resting potential of the innervated muscle was unaffected (Table 1). However, TTX had a marked hyperpolarizing effect on muscles which were denervated for 3 and 7 days, altering them by 5.3 mV and 7.4 mV respectively. This hyperpolarizing effect was less marked at 14 days (3.9 mV) and was totally absent at 21 days after denervation (Table 1).

Denervation also produced alterations in some properties of directly-elicited action potentials (Table 1 and Fig. 1). The rate of rise of the action potential ( $dV/dt$ ) was decreased from a control value of  $671 \pm 22$  V/s to  $536 \pm 29$  V/s by the third day after nerve section. At later times after denervation the  $dV/dt$  continued to decrease reaching a low value of  $331 \pm 9$  V/s at 21 days. However, the rate of

decrease of  $dV/dt$  was significantly greater during early denervation (0–3 days) than at later times. The overshoot potential was unchanged from the control value up to and including 14 days after denervation but was reduced at 21 days (Table 1). In addition, the repolarization rate was decreased and the duration of the action potential was increased after denervation (Fig. 1). Although it was not quantitated, a larger current was necessary to hyperpolarize the membrane to  $-90$  mV at 3 days of denervation than for innervated muscle or for denervated for longer periods.

A dose-response curve for TTX was obtained from both the normal and the denervated muscles of 2) TTX rapidly reduced the  $dV/dt$  of the innervated muscles at moderate concentrations and completely blocked the action potential at  $10^{-6}$  M. In contrast, all the denervated muscles were partially resistant to the action of TTX. However, despite the differences in the initial  $dV/dt$  for the denervated muscles, the action of increasing concentrations of TTX was similar throughout the time course of the study.

In  $10^{-6}$  M TTX, all fibers sampled generated action potentials at 3 days after denervation. The  $dV/dt$  and overshoot potential in  $10^{-6}$  M TTX increased up to and including 7 days after denervation (Table 1 and Fig. 1). After this time, however, both the  $dV/dt$  and overshoot potential in  $10^{-6}$  M TTX decreased reaching steady values between 14 and 21 days after denervation (Table 1 and Fig. 1).

## DISCUSSION

The fall in resting membrane potential after denervation observed in this study was similar to

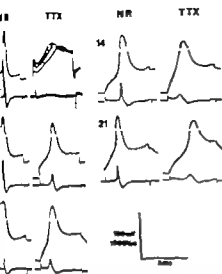


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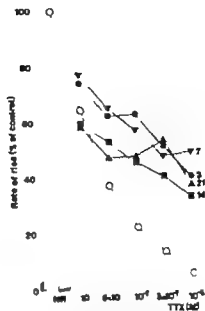


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## DISCUSSION

The fall in resting membrane potential after denervation observed in this study was similar to that reported by others (Thesleff 1970; Sellin 1971; Thesleff & Sellin 1972).

# Effects of thyroliberin and 4-aminopyridine on action potentials and prolactin release and synthesis in rat pituitary cells in culture

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SAND O. HAUG & GAUTVIK, K. M. Effects of thyroliberin and 4-aminopyridine on action potentials and prolactin release and synthesis in rat pituitary cells in culture. *Acta Physiol Scand* 1980; 108: 247-252. Received 8 June 1979. Departments of Physiology, Veterinary College of Norway, Oslo; Hormone and Isotope Laboratory, Alster Hospital, Oslo; and Institute of Physiology, University of Oslo, Norway.

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**Key words:** Anterior pituitary action potential, thyroliberin, 4-aminopyridine, prolactin release, prolactin synthesis.

Intracellular  $Ca^{2+}$  is a prerequisite for the stimulatory effect of thyroliberin (TRH) on prolactin (PRL) release from cultured rat pituitary tumour cells (GH cells) (Kautvik et al. 1977, 1979; Tashjian et al. 1978). A certain fraction of both normal anterior pituitary cells and clonal GH cells displays an increased frequency of partly  $Ca^{2+}$  dependent action potentials in TRH containing medium (Kikukawa 1973; Tanishevich & Douglas 1977). Furthermore, Tanishevich et al. (1979) observed prolonged action potentials in TRH solution. By recording from the same cell before and during TRH stimulation, we have been able to confirm that TRH is responsible for these effects. Both the increased firing rate and the prolongation of the action potential will increase the  $Ca^{2+}$  influx. However, no direct evidence exists in favour of a functional relationship between the TRH-stimulated action potentials and the TRH-mediated hormone release.

TRH could increase the resting membrane permeability to  $Ca^{2+}$  and thus induce  $Ca^{2+}$ -triggered hormone release independently of the action potentials. Such a possibility may be tested by employing drugs which exclusively affect voltage dependent permeability changes. The effects of 4-aminopyridine (4AP) on the action potentials and on the release and synthesis in the GH cells are described in the present paper. This drug has no effect on resting membrane permeabilities but inhibits the late K<sup>+</sup> current in a variety of excitable cells (Pelhate & Pichon 1974; Gillespie & Hutter 1975; Lindis et al. 1976; Ubricht & Wagner 1976; Yeh et al. 1976; Molgo et al. 1977). We find that 4AP both increases the spontaneous firing rate and prolongs the action potentials in GH cells, thus mimicking effects of TRH. If TRH stimulates hormone release due to its effects on the action potentials, 4AP should show a similar effect on the release. If TRH

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# Effects of thyroliberin and 4-aminopyridine on action potentials and prolactin release and synthesis in rat pituitary cells in culture

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Effects of thyroliberin (TRH) and 4-aminopyridine (4AP) were studied on prolactin (PRL) secreting rat pituitary tumour cells in culture (GH<sub>4</sub> cells). Intracellular recordings obtained from the same cell before and during TRH stimulation showed this peptide to increase the spontaneous firing frequency and prolong the Ca<sup>2+</sup>-dependent action potentials. These effects were unmasked by 4 AP which acts by interfering selectively with voltage dependent ionic channels without affecting resting membrane properties. Optimal doses of TRH and 4AP approximately doubled the release of PRL. In contrast, TRH increased PRL synthesis 1.9-fold while 4AP had no effect. Increased PRL synthesis is thus not a direct consequence of the hormone release. We conclude that TRH and 4AP both stimulates PRL release via the facilitating effects on the action potentials. TRH has additional intracellular effects which lead to increased synthesis of the hormone. The effects of TRH responsible for stimulation of PRL synthesis are not causally related to the impulse activity of the surface membrane of the cell.

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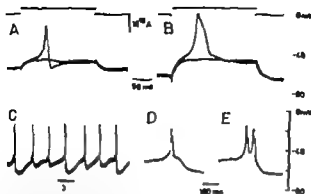


Fig. 2 Electrophysiological effects of 4-aminopyridine (4AP). (A) All or none action potential induced by depolarizing current injection in control solution. (B) Corresponding recording from different cell in solution containing  $1.5 \times 10^{-4}$  M 4AP. Note the extended falling phase and absence of after-hyperpolarization. (C) Spontaneous action potentials in  $5 \times 10^{-4}$  M 4AP solution. Recording from the same cell at higher sweep speed shows single action potentials with pronounced shoulder on the repolarizing phase (D) or action potentials with double peaks (E).

and stored PRL, as therefore measured as the total of hormone which accumulated in the medium in 20 min of incubation after addition of 4AP or TRH. At the end of the incubation periods PRL release was tested by transferring the tubes to ice-water and centrifuging (3000g) at 0°C for 5 minutes. Extracellular PRL, measured by radioimmunoassay (Haug & Olsrvik 1977). All samples from one experiment were included in one assay series, and the determinations were performed in triplicate. The cell protein content of the tubes was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

**PRL synthesis.** PRL synthesis was measured in antibody calorimeters as the amount of hormone which accumulated in the culture medium during 48 h. The intracellular levels of PRL are very small, and there is no intracellular degradation of PRL (Haug et al. 1977). Furthermore, PRL is stable in the culture medium under normal conditions for as long as 48 h (Teschner et al. 1970). The extracellular amount of PRL therefore represents more than 95% of the total amount of hormone synthesized by the cells during 48 h (Haug et al. 1977). At the end of the treatment period the culture medium was collected and the cells were scraped into 0.15 M NaCl. The washed 4 tubes. Medium and cells were stored at -20°C until assays for PRL and cell protein were performed.

## RESULTS

### Electrophysiological recordings

#### TRH effects

Successful experiments were characterized by membrane potentials and input resistances of 40–65 MΩ and 400–1400 MΩ, respectively. Action potentials could be generated in these cells either by

depolarizing current injection (Fig. 2A) or at the termination of a hyperpolarizing current pulse (Fig. 1A). The ionic basis for the action potential and the general electrophysiological properties of the GH cell membrane have been described in detail elsewhere (Ozawa et al. 1979; Ozawa & Miyazaki 1979).

Electrically induced action potentials were recorded from 17 cells in this particular series of experiments. None of the cells fired action potentials spontaneously. Only 7 of the cells were sufficiently durable to exhibit stable membrane properties for the period required for TRH stimulation and observation of possible effects of TRH. TRH clearly altered the electrical membrane properties of 3 of these cells. Fig. 1A shows an action potential evoked at the termination of a hyperpolarizing current pulse before TRH stimulation of one of these cells. The stimulation pipette was lowered through the solution during the time between record A and B. Record B was obtained just after the tip of the TRH pipette was positioned at a distance of 40 μm from the cell and before forced ejection of TRH had occurred. It is seen that the shape of the action potential was clearly altered even at this stage, indicating that TRH had leaked out from the pipette. The most obvious change was a prolongation of the falling phase of the action potential. TRH was then injected into the solution and after about 20 s the cell displayed spontaneous action potentials as seen in Fig. 1C. The firing rate was about 0.7 Hz in this



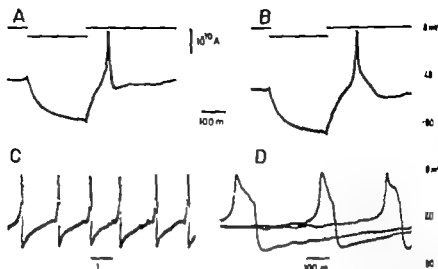


Fig. 1 Electrophysiological effects of thyrotropin releasing hormone (TRH). (A) Action potential induced in control solution by anodal break stimulation. (B) Corresponding recording immediately after positioning of the TRH pipette close to the cell. Note the retarded falling phase of the action potential. (C) Spontaneous activity recorded 70 s after ejection of TRH. (D) Three superimposed sweeps showing the prolongation of the spontaneous action potentials relative to the electrically evoked action potentials in control solution. Same sweep speed as in A and B. All recordings are from the same cell.

causes release through different mechanisms. 4AP would not be expected to stimulate hormone release. The potency of 4AP to facilitate PRL release from GH<sub>3</sub> cells is similar to that of TRH. However, TRH also stimulates PRL synthesis, whereas 4AP does not have this biological action.

## MATERIAL AND METHODS

**Cell cult.** The establishment and culture of the GH<sub>3</sub> strain of rat pituitary tumour cells have been described previously (Tashjian et al. 1968). The cells were grown as monolayers in plastic dishes containing Ham F 10 medium supplemented with 15% horse serum and 5% fetal calf serum at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The medium was changed every 2–3 days. Antibiotics were added to the culture medium and these did not influence cell growth or hormone synthesis (Haug & Gautvik 1976).

**Electrophysiological experiment.** The recordings were obtained 3–10 days after subculture. Just prior to an experiment the F 10 medium was removed and replaced with the following saline (mM): NaCl 140, KCl 5, CaCl<sub>2</sub> 10, MgCl<sub>2</sub> 1.3, glucose 10, buffered by Tris HCl 5 to pH 7.4. The experiments regarding the effect of TRH and the initial recordings in 4AP solution were performed in saline containing 70 mM CaCl<sub>2</sub> (1.5 mM NaCl) since high Ca concentration is known to facilitate stable penetrations of GH<sub>3</sub> cells (Kikukawa 1974). TRH was administered through a micropipette containing 1 μM TRH and with a tip diameter between 5 and 8 μm. The tip was kept just above the surface of the solution to avoid that TRH affected the cells due to leakage from the tip during the periods between successful electrode penetrations. When a stable recording was achieved from a cell displaying

electrical excitability the TRH pipette was slowly retracted through the solution by means of a hydraulic drive. The tip of the pipette was positioned at a distance of 30–50 μm from the cell and TRH was slowly projected into the solution. It proved difficult to obtain stable penetration for the time required for the period. The experiments involving 4AP were therefore performed with those obtained in saline containing between 5 and 3 × 10<sup>-4</sup> M 4AP.

The cells were viewed through an inverted microscope during the experiments which were conducted at 37°C. The glass microelectrodes used for intracellular recording were filled from behind by capillary action with 1 M KCl or 5 M K-acetate adjusted to pH 7 with acetic acid. The electrode resistance ranged between 10 and 250 MΩ. The electrode was connected to a preamplifier with a facility for injecting current into the recording electrode using a bridge circuit (Bio Electronics Lab, AM 1). The input resistance of the preamplifier was more than 10<sup>12</sup> Ω and the input current was less than 10<sup>-14</sup> A.

**PRL release.** PRL release was measured in GH<sub>3</sub> growing in either the late logarithmic or the early stationary phase of growth (6–10 days after subculture). At the beginning of the experiment the F 10 medium was removed and the cells scraped into Neuman-Tyrell's serumless medium and washed three times. The cells were then resuspended in serumless medium and 1 μl aliquots of the cell suspension were added to tubes with or without 4AP or TRH. The subsequent incubation was carried out at 37°C in a shaking water bath.

Previous experiments have shown that when radioactive amino acids are added to the culture medium, synthesized radioactive PRL will appear in the medium later (Gautvik & Kniz 1976). Release of labeled

ation, and our data support the study by us et al (1979) who compared recordings from GH<sub>3</sub> cells exposed to various solutions. The conclusions regarding the electrophysiological effects of TRH on GH<sub>3</sub> cells are that this peptide causes spontaneous firing and prolongs the action potential. Our recordings show that both these effects are clearly visible within 30 s, which is the time required for 2/3 of maximal binding of TRH to membrane receptors (Gautvik et al 1979b). It has been suggested that the GH<sub>3</sub> cultures consist of sub-populations secreting either PRL or growth hormone (Gautvik & Kriz 1976). This could explain why less than half of the studied cells were clearly stimulated by TRH. Furthermore, our recordings are obtained from unsynchronized cultures and cells may have different membrane properties depending on the phase of growth.

The observed electrophysiological effects of TRH on GH<sub>3</sub> cells mimic certain of the effects attributed for TRH. Both compounds 1) increase the spontaneous firing and 2) prolong the action potential. The action of 4AP is compatible with a reduction of the late K<sup>+</sup> current. This will both retard the repolarizing phase of the action potential and delay the after-hyperpolarization. A beginning depolarization below threshold will furthermore be followed by a reduced outward-going rectification. The probability of spike initiation will thus increase.

The close similarity between the electrophysiological effects on GH cells caused by TRH and 4AP does not necessarily reflect a common mode of action for the two substances. The increased firing rate and prolonged action potentials induced by TRH may for instance be caused by a direct action on the voltage dependent Ca<sup>2+</sup> permeability. It has recently become clear that several neurotransmitters with presynaptic action exert their effects by interfering with the voltage dependent Ca<sup>2+</sup> current in the presynaptic terminal without altering resting membrane properties. Presynaptic facilitation is thus associated with an increased Ca<sup>2+</sup> permeability and prolonged action potentials (Klein & Kandel 1978; Peñáz & Carpenter 1978), whereas reduced Ca<sup>2+</sup> current and shortened action potentials seem to be responsible for presynaptic inhibition (Dunlap & Fischbach 1978; Lodge et al 1979). Klein & Kandel (1978) suggest that serotonin causes presynaptic facilitation by increasing a cyclic AMP mediated increase of a voltage

sensitive Ca<sup>2+</sup> current in the terminals of sensory neurons in *Aplysia*. The action of TRH on the GH cell membrane might be related to such a mechanism and it has been shown that an early effect of TRH on GH cells involves cyclic AMP formation (Dannies et al 1976; Gautvik et al 1977, 1979a).

In addition to the well established effect of 4AP on the late K<sup>+</sup> current it has recently been suggested that this drug may also have a direct effect on the voltage sensitive Ca<sup>2+</sup> channels in some excitable membranes (Jankowska et al 1977; Lundh & Theleff 1977; Galindo & Rudomin 1978). However, the resting membrane potential and resistance are not altered by 4AP (Pelhate & Picbon 1974; Molgo et al 1977). We therefore conclude that 4AP facilitates PRL release through a selective effect on the action potentials; an explanation in agreement with previous reports of the effect of 4AP on neurotransmitter release (Lindén et al 1976; Jankowska et al 1977; Krippekar et al 1977; Lundh & Theleff 1977; Molgo et al 1977; Galindo & Rudomin 1978).

4AP had no effect on PRL synthesis and cell growth in contrast to the marked stimulation of PRL release. This stimulation was similar to that induced by TRH. Furthermore, 4AP which acts by selectively interfering with voltage dependent ionic channels without affecting resting membrane properties mimics certain of the effects of TRH on the action potentials. These observations support the idea that TRH enhances PRL release from GH cells via its facilitating effects on the Ca<sup>2+</sup> dependent action potentials, both regarding firing rate and spike duration. Moreover, since PRL secretion was stimulated by 4AP without a concomitant increase in synthesis, the latter process is not necessarily a consequence of hormone release. Therefore TRH regulation of PRL secretion and synthesis is probably exerted through independent mechanisms as suggested by Gautvik et al. (1978).

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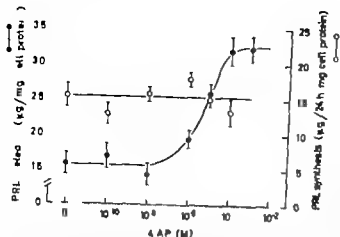


Fig. 3 Effects of 4AP on PRL release and synthesis. The treatment periods were 20 min for the release and 7 days for the synthesis experiments. Data are given as mean values  $\pm$  S.E. of quadruplicates. Values significantly different from controls ( $P < 0.05$ ) are marked with asterisks. TRH ( $10^{-8}$  M) caused a 1.9-fold increase in PRL release and synthesis (data not shown).

cell. Fig. 1 D presents 3 superimposed recordings at the same sweep speed as in records A and B, and it is seen that the spontaneous action potentials were markedly prolonged compared to the electrically evoked potentials prior to TRH stimulation. The remaining two cells which were visibly affected by TRH showed a similar behaviour to that presented in Fig. 1. However, one of these cells was silent even after TRH stimulation, although the electrically evoked action potentials showed a prolonged falling phase.

#### Electrophysiological registrations of 4AP effects

Action potentials induced by current injections were recorded from 12 cells in  $1-3 \times 10^{-3}$  M 4AP solutions. In the control solution action potentials were followed by a pronounced after hyperpolarization (Fig. 2A) but this was abolished in the presence of 4AP (Fig. 2B). Several cells in 4AP solution showed in addition a clear prolongation of the repolarization phase of the action potential as displayed in Fig. 2B. Both these alterations of the action potentials are consistent with an inhibitory effect of 4AP on the late  $K^+$ -current which is well developed in GH<sub>3</sub> cells (Ozawa et al. 1979).

Spontaneous action potentials were not observed in these initial experiments which were performed in 20 mM  $Ca^{2+}$  solution. High  $Ca^{2+}$  concentrations are known to reduce the excitability of cell membranes (Frankenhaeuser 1957) and the  $Ca^{2+}$  concentration was therefore decreased to 10 mM in the

remaining recordings. Eleven cells were successfully penetrated in such solution containing  $5 \times 10^{-4}$  M 4AP, and 4 of these cells displayed spontaneous action potentials with a firing frequency of 0.1–1 Hz. Fig. 2C presents a recording from a spontaneously active cell and Fig. 2D shows the shape of the action potentials at higher sweep speed. A pronounced shoulder is evident on the repolarization phase of the action potential, and a second action potential was occasionally initiated from the retarded falling phase as seen in Fig. 2E. Spontaneous activity was not observed in any of the 9 cells successfully impaled in control solution, and single and double peaks were never seen on the electrically induced action potentials in these cells. 10 mM  $Ca^{2+}$  is an unphysiologically high concentration, and extracellular recordings from GH<sub>3</sub> cells at normal  $Ca^{2+}$  levels have shown the majority of cells to fire action potentials spontaneously (Klibon 1975). However, we did not achieve stable penetrations at  $Ca^{2+}$  concentrations below 10 mM.

#### Effects of 4AP on PRL release and synthesis

Secretion of PRL was measured after 20 min incubation with 4AP in concentrations of  $10^{-4}$ – $10^{-2}$  M. A dose-dependent effect was observed, with maximal stimulation of PRL secretion ( $^3$  fold) at  $10^{-4}$  M as shown in Fig. 3. It has previously been shown that  $10^{-4}$  M TRH gives maximal stimulation of the PRL release from GH<sub>3</sub> cells (Dumortier-Tashjian 1974), and the effect of TRH at this concentration was therefore measured in the same experiment for comparison. Incubation with  $10^{-4}$  M TRH for 70 min increased the PRL release from a basal level of  $1.6 \pm 0.15$   $\mu$ g/mg cell protein to  $3.1 \pm 0.16$   $\mu$ g/mg cell protein (S.E.  $n=4$ ). Samples were also taken already after 5 min of treatment with  $10^{-4}$  M 4AP and  $10^{-4}$  M TRH, and a significant stimulation ( $P < 0.05$ ) of PRL release was observed for both compounds. 4AP is thus to a large extent able to duplicate the effect of TRH on PRL release.

In contrast to the effect on PRL release, 4AP induced no change in the rate of hormone synthesis (Fig. 3). However, the PRL synthesis was increased 1.9 fold in  $10^{-4}$  M TRH solution. There was no effect of 4AP on cell growth at any dose level.

#### DISCUSSION

The presented intracellular effects of TRH were observed on the same cells before and during TRH

# Sensitization of the rat parotid gland to secretagogues following either parasympathetic denervation or sympathetic denervation or decentralization

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The sensitivity of the rat parotid gland to chemical agents was examined 2-3 weeks after either parasympathetic denervation or sympathetic denervation or decentralization. The parasympathetically denervated gland was markedly sensitized towards methacholine: a nonspecific supersensitivity mainly mediated via  $\alpha$ -adrenoceptors was also demonstrated. The sympathetically denervated gland had developed supersensitivity which was both of the pre- and the postjunctional type: it was predominantly of the former type to noradrenaline and adrenaline, and entirely so to phenylephrine. It was of the latter type to isoprenaline and also to methacholine. The sympathetically decentralized gland was stably sensitized towards noradrenaline, adrenaline and isoprenaline. The postjunctional supersensitivity developed towards the sympathomimetic drugs after sympathetic decentralization was of about the same magnitude as that observed after sympathetic denervation, this type of supersensitivity was mainly mediated via  $\beta$ -adrenoceptors.

**Key words:** Parasympathetic denervation, sympathetic denervation or decentralization, pre- and postjunctional supersensitivity, preponderance for either  $\alpha$ - or  $\beta$ -adrenoceptor mediated sensitization, parotid gland, rat.

Studies on the activity of the acetylcholine-forming enzyme, choline acetyltransferase and its dependence on the traffic of nerve impulses in the parotid gland of the rat has been found to be a useful model organ (see Ekström 1978). The sensitivity of this gland to chemical stimuli has in some investigations been used to indicate changes in the traffic of sensory impulses to the gland. Thus in rats kept on a liquid diet, showing a decreased impulse traffic to gland cells were more sensitive to the parasympathetic substance methacholine than those of rats kept on a dry and bulky diet (Ekström & Temelkova 1977). Further loss or reduction of the traffic caused by prolonged treatment with a ganglion blocking drug sensitized the gland cells to methacholine (Ekström & Landmark 1977, 1978). In contrast to the submaxillary gland of the rat (Ohlin & Wä, Åsberg & Ekström 1979), few observations have been made on the effect of denervation or decentralization on the sensitivity of the parotid gland of this species to secretagogues (see Alm &

Ekström 1976). In the present study the sensitivity of the parotid gland to parasympathomimetic and sympathomimetic drugs has been examined following either parasympathetic denervation or sympathetic denervation or decentralization. The parotid gland of the rat like the submaxillary gland, belongs to those few salivary glands that have a rich sympathetic nerve supply (Norberg & Olson 1965, Alm & Ekström 1977) which on electrical stimulation causes a lively flow of saliva (Thulin 1976).

## METHODS

92 adult female rats of Sprague Dawley strain bred at the Department of Physiology were used. The animals weighed at the end of the experimental period  $17 \pm 4$  g (mean  $\pm$  S.E.). The operations were made under ether anaesthesia with the aid of a dissecting microscope. Parasympathetic denervation of the parotid gland was achieved by avulsion of the acriculo-temporal nerve. Sympathetic denervation by removal of the superior cervi-

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2. Threshold (minimum) doses of methacholine noradrenaline adrenalin phenylephrine and isoprenaline evoking secretory responses in the parotid gland either parasympathetically denervated or arterially denervated or decentralized 2-3 weeks in advance and in the contralateral parotid gland

(\*) Isoprenaline (D641) 1.5  $\mu\text{g/kg}$  was given intravenously previous to the test doses. Number of observations is in brackets. Values are mean  $\pm$  S.E.

	Methacholine ( $\mu\text{g/kg}$ )	Noradrenaline ( $\mu\text{g/kg}$ )	Adrenaline ( $\mu\text{g/kg}$ )	Phenylephrine ( $\mu\text{g/kg}$ )	Isoprenaline ( $\mu\text{g/kg}$ )
symp. den.					
operated gland	0.14 $\pm$ 0.01 (8) <sup>***</sup>	4.9 $\pm$ 1.0 (7)*	4.1 $\pm$ 0.6 (7) <sup>**</sup>	31.4 $\pm$ 5.3 (14)*	2.7 $\pm$ 1.0 (9)†
contralateral gland	0.84 $\pm$ 0.11 (8)	15.0 $\pm$ 2.4 (7)	9.3 $\pm$ 0.7 (7)	72.9 $\pm$ 7.8 (14)	2.1 $\pm$ 0.4 (9)
den.					
operated gland	0.73 $\pm$ 0.09 (18) <sup>**</sup>	0.65 $\pm$ 0.06 (14) <sup>***</sup>	2.6 $\pm$ 0.5 (11) <sup>***</sup>	16.7 $\pm$ 2.1 (16) <sup>**</sup>	0.94 $\pm$ 0.16 (10)
contralateral gland	1.22 $\pm$ 0.24 (18)	6.9 $\pm$ 0.8 (14)	8.6 $\pm$ 1.8 (11)	90.0 $\pm$ 0.0 (6)	1.6 $\pm$ 0.19 (10)
den. D641					
operated gland	Not tested	0.71 $\pm$ 0.11 (8)*	4.7 $\pm$ 0.3 (9)†	32.0 $\pm$ 6.2 (10)†	Not tested
contralateral gland		1.1 $\pm$ 0.4 (8)	5.0 $\pm$ 0.0 (9)	115 $\pm$ 6.0 (10)	
den.					
operated gland	1.8 $\pm$ 0.1 (11)*	4.3 $\pm$ 0.4 (12) <sup>**</sup>	5.8 $\pm$ 0.6 (12)*	51.7 $\pm$ 5.1 (12)†	0.64 $\pm$ 0.16 (5) <sup>**</sup>
contralateral gland	1.1 $\pm$ 0.1 (11)	8.3 $\pm$ 1.3 (12)	7.5 $\pm$ 0.8 (12)	54.4 $\pm$ 4.2 (12)	1.4 $\pm$ 0.2 (5)

\*  $P < 0.05$  \*\*  $P < 0.01$  \*\*\*  $P < 0.001$  when the operated gland is compared with the contralateral gland.

had roughly it was half of that found in contralateral gland. For the  $\beta$ -adrenoceptor blocking drug isoprenaline no difference in the threshold dose between operated and contralateral gland was observed there was however a significant difference ( $p < 0.01$ ) to be found when the selected  $\beta$ -adrenoceptor stimulator H 80/6 was tested (operated gland 6.0 $\pm$ 1.1  $\mu\text{g/kg}$ ; contralateral gland 18.6  $\mu\text{g/kg}$ ; number of observations, 11). The amount of saliva secreted per gland in response to various doses of methacholine noradrenaline and phenylephrine was larger from the operated than from the contralateral gland. As to the response evoked by suprathreshold doses of the adrenoceptor stimulating drugs no difference between operated and contralateral gland was found in connection with isoprenaline whereas the results with H 80/6 varied. To exemplify methacholine 2.15  $\mu\text{g/kg}$  caused the operated gland to secrete 11.1 ( $n=8$ ) and 14.2 $\pm$ 1.0 ( $n=8$ ) mg saliva, and the contralateral gland 1.8 $\pm$ 0.5 ( $n=8$ ) and 4.2 $\pm$ 0.4 ( $n=8$ ) mg saliva. In response to noradrenaline 20  $\mu\text{g/kg}$  the operated gland secreted 1.9 $\pm$ 0.6 ( $n=7$ ) and the contralateral gland 0.5 $\pm$ 0.2 ( $n=7$ ) mg saliva. And to the same dose of adrenaline 3.5 $\pm$ 0.8 ( $n=7$ ) and 1.1 $\pm$ 0.3 ( $n=7$ ) mg saliva, respectively. With isoprenaline 5 and 10  $\mu\text{g/kg}$  the operated gland secreted 0.6 $\pm$ 0.1 ( $n=9$ ) and 1.4 $\pm$ 0.4 ( $n=9$ ) mg saliva, while the contralateral gland secreted 0.8 $\pm$ 0.2

( $n=9$ ) and 1.5 $\pm$ 0.3 ( $n=9$ ) mg saliva, in response to 11.80/6, the amount of saliva secreted at a dose of 20  $\mu\text{g/kg}$  was larger ( $p < 0.001$ ) from the operated gland (1.6 $\pm$ 0.2 mg saliva,  $n=10$ ) than from the contralateral gland (0.9 $\pm$ 0.1 mg saliva,  $n=10$ ) while at the level of 50  $\mu\text{g/kg}$  there was no significant difference between the glands (operated gland, 2.6 $\pm$ 0.5 mg saliva, contralateral gland: 1.9 $\pm$ 0.2 mg saliva, number of observations 9). When relating the amount of saliva secreted after administration of isoprenaline to the dry weight of the gland a significantly ( $p < 0.01$ ) larger figure is obtained for the operated than for the contralateral gland at the dose level of 10  $\mu\text{g/kg}$ , being 0.0560 $\pm$ 0.007 ( $n=9$ ) and 0.0376 $\pm$ 0.0067 ( $n=9$ ) mg saliva per mg gland. The secretion evoked by isoprenaline was very viscous. In some rats the injection of isoprenaline was therefore repeated after a previous injection of methacholine methacholine causing a watery secretion, was used to fill up the duct system in an attempt to reduce obstruction to the salivary flow. However the amount of saliva appearing at the tip of the cannula was the same as before the injection of methacholine.

**Sympathetic de-ervation** The threshold doses for all the drugs tested were lower in the operated than in the contralateral gland. In the operated gland the threshold dose for methacholine was about half of that in the contralateral gland, for noradrenaline

Table 1 Wet and dry weights of the parotid gland either parasympathetically denervated or not, centrally denervated or decentralised 2-3 weeks in advance and those of the contralateral parotid gland. Number of observations is given in brackets. Values are mean  $\pm$  S.E.

	Wet weight (mg)	Operated gland/ contralateral gland (%)	Dry weight (mg)	Operated gland/ contralateral gland (%)
Parasymp den				
Operated gland (18)	85.6 $\pm$ 3.4	39.6 $\pm$ 2.2	4.6 $\pm$ 1.0	39.9 $\pm$ 2.0**
Contralateral gland (18)	146.0 $\pm$ 7.1		4.0 $\pm$ 1.0	
Symp den				
Operated gland (50)	170.7 $\pm$ 3.2	87.4 $\pm$ 2.1	3.3 $\pm$ 1.0	86.1 $\pm$ 7.0**
Contralateral gland (50)	139.3 $\pm$ 3.3		3.8 $\pm$ 1.1	
Symp dec				
Operated gland (4)	116.7 $\pm$ 4.6	89.8 $\pm$ 2.7*	33.0 $\pm$ 1.0	91.4 $\pm$ 3.5*
Contralateral gland (24)	131.0 $\pm$ 5.4		36.1 $\pm$ 1.6	

$P < 0.05$        $P < 0.001$  when the operated gland is compared with the contralateral gland.

cal ganglion and sympathetic decentralization by cutting the cervical sympathetic nerve trunk. The surgical procedure was made unilaterally; the contralateral gland served as control 2-3 weeks after surgery. The animals were anaesthetized with chloralose (100 mg/kg) through a femoral venous cannula after induction with ether. The animals were provided with a tracheal cannula and the body-temperature was checked with a rectal thermometer. On both sides the duct of the parotid gland was exposed near its entrance in the mouth and cannulated using a fine glass cannula, which gave about 115 drops from 1 ml distilled water. Saliva appearing at the tip of the cannula was collected on filter paper and weighed. The amount of saliva secreted was expressed in mg saliva per gland or in mg saliva per mg dry weight of the gland. To estimate threshold dose and the submaximal secretory responses a series of standard doses of methacholine chloride (usually in the range of 0.01-10  $\mu$ g/kg), noradrenaline bitartrate (0.1-20  $\mu$ g/kg), adrenaline bitartrate (0.5-70  $\mu$ g/kg), phenylephrine hydrochloride (1-100  $\mu$ g/kg) and isoprenaline sulphate (0.5-10  $\mu$ g/kg) were injected through the femoral cannula. In some experiments the selective  $\beta$ -adrenoceptor stimulating drug H 80/67 was also used (see Ekström 1979) since the drug has a long duration of action the amount of saliva secreted was expressed per 5 min. Because of deterioration of the preparation the number of drugs tested on each rat was usually restricted to two or three. After the injection of the largest dose of the sympathomimetic drugs a respiratory arrest was often observed except for isoprenaline and H 80/67. In such a case artificial respiration was temporarily given by blowing air into the tracheal cannula. When desmethyldimethylamine hydrochloride 1.5 mg/kg was used to inactivate the neuronal amine pump the drug was injected slowly i.v. over a period of 10 min. 10 min thereafter the first of the standard doses was injected.

At the end of the acute experiment the parotid gland was removed, cleaned, pressed between gauze pads and weighed before (wet weight) and after heating to 105-110°C for 48 h (dry weight).

Student's *t* test was used, paired comparisons were made between the operated gland and the contralateral unoperated gland. The 0.05 level of probability was considered significant.

## RESULTS

### Gland weights

After parasympathetic denervation both the wet and the dry weight of the operated gland was found to be about 60% of the contralateral gland (Table 1). The wet and the dry weight of the sympathetically denervated gland was 87 and 86% of the contralateral gland, respectively; the corresponding percentage figures for the sympathetically decentralized gland were 90 and 93 (Table 1). There was a significant difference between the weights (wet and dry) of the sympathetically denervated gland and those of the sympathetically decentralized gland when comparisons were made on percentage to (operated gland as percent of contralateral gland).

### Secretory responses

Threshold doses for the drugs are shown in Table 2 and the amounts of saliva secreted in response to submaximal doses in Figs. 1-4.

**Parasympathetic denervation.** In the operated gland the threshold dose for methacholine was one-sixth for noradrenaline one-third and for isoprenaline half of that needed to evoke secretion from the contralateral gland. The threshold dose for  $\alpha$ -adrenoceptor stimulating drug phenylephrine was also lower in the operated gland than in the contralateral gland.

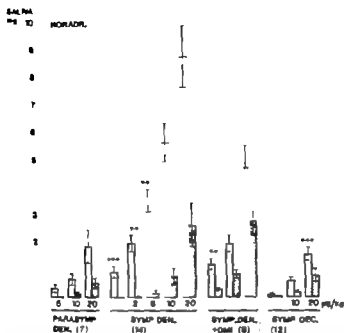


Fig. 2 Noradrenaline. Secretory responses, in mg saliva, of various intravenous doses, in  $\mu\text{g/kg}$ , of noradrenaline from parotid glands: unoperated (hatched columns) or either sympathetically denervated or sympathetically denervated or decentralised, 3 weeks in advance (open columns). DMF indicates the presence of desmethylnormetazepam. For further explanations see Fig. 1.

ated gland secreted larger amount of saliva than the contralateral gland although the difference was not as big as marked as in the group of rats where these drugs were tested without the presence of desmethylnormetazepam. For instance noradrenaline, 2 and 5  $\mu\text{g/kg}$ , evoked a secretion of  $1.8 \pm 0.3$  ( $n=8$ ) and  $4.8 \pm 0.5$  ( $n=8$ ) mg saliva from operated gland and from the contralateral gland  $1.0 \pm 0.1$  ( $n=8$ ) and  $2.8 \pm 0.5$  ( $n=8$ ) mg of saliva, and adrenaline, 5 and 10  $\mu\text{g/kg}$ , evoked a secretion of  $1.8 \pm 0.3$  ( $n=9$ ) and  $2.1 \pm 0.2$  ( $n=9$ ) mg saliva from operated gland, and  $1.0 \pm 0.1$  ( $n=9$ ) and  $1.7 \pm 0.1$  ( $n=9$ ) mg saliva from the contralateral gland. In a group of rats neither noradrenaline nor adrenaline was tested at the dose level of 20  $\mu\text{g/kg}$ . In response to phenylephrine 50 and 100  $\mu\text{g/kg}$ , the amount of saliva secreted from the operated gland did not differ significantly from that secreted from the contralateral gland, neither were there any differences to be found when the comparisons between operated and contralateral gland to be based on mg of saliva secreted per mg dry tissue weight. In the rats treated with desmethylnormetazepam the contralateral

glands secreted larger amount of saliva than the corresponding glands in the rats not treated with this drug, as can be seen in the Figs. 4.

**Sympathetic dec. neutralization** The threshold dose for methacholine in the separated gland did not differ significantly from that in the contralateral gland neither was there any difference to be found for phenylephrine. For noradrenaline and isoprenaline the threshold dose in the operated gland was about half of that in the contralateral gland. For adrenaline the threshold dose was also lower in the operated than in the contralateral gland, although the difference in threshold dose between the glands was not as big as that found for noradrenaline and isoprenaline. In response to submaximal doses of methacholine and phenylephrine a difference between operated and contralateral gland could only be demonstrated at some dose levels provided the amount of saliva secreted was related to the tissue weight. Thus at 5 and 10  $\mu\text{g/kg}$  of methacholine the operated gland secreted  $0.1364 \pm 0.0139$  ( $n=11$ ) and  $0.2683 \pm 0.0213$  ( $n=11$ ) mg saliva per mg gland, while the contralateral gland secreted  $0.1099 \pm$



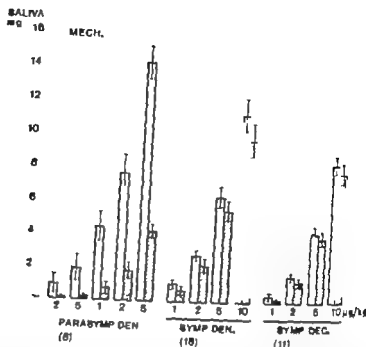


Fig 1 Methacholine secretory responses in mg saliva of various intravenous doses, in  $\mu\text{g}/\text{kg}$ , of methacholine from parotid glands: unoperated (hatched columns) or either parasympathetically denervated or sympathetically denervated or decentralized 3 weeks in advance (open columns). The column and its vertical bar is mean  $\pm$  S.E. Number of observations is indicated in brackets. Comparison was made between the operated gland and its contralateral unoperated gland. In the case of a significant difference this is shown as follows:  $P < 0.05$   $P < 0.01$   $P < 0.001$ .

one-tenth for adrenaline and phenylephrine about one third and for isoprenaline roughly half of that in the contralateral gland. To all the drugs the secretion in response to submaximal doses was larger from the operated than from the contralateral gland although to methacholine the difference was not very conspicuous. The amount of saliva secreted in response to methacholine 2 and 5  $\mu\text{g}/\text{kg}$  was from the operated gland  $2.8 \pm 0.3$  ( $n=18$ ) and  $6.3 \pm 0.6$  ( $n=18$ ) mg saliva and from the contralateral gland  $2.2 \pm 0.4$  ( $n=18$ ) and  $5.5 \pm 0.6$  ( $n=18$ ) mg saliva. Of the sympathomimetic drugs the most marked differences were observed in connection with noradrenaline, adrenaline and phenylephrine: e.g. noradrenaline 10 and 20  $\mu\text{g}/\text{kg}$  evoked a secretion of  $5.6 \pm 0.7$  ( $n=14$ ) and  $8.7 \pm 1.1$  ( $n=14$ ) mg saliva from the operated gland and  $0.8 \pm 0.3$  ( $n=14$ ) and  $2.6 \pm 0.8$  ( $n=14$ ) mg saliva from the contralateral gland; adrenaline 10 and 20  $\mu\text{g}/\text{kg}$   $2.6 \pm 0.5$  ( $n=11$ ) and  $5.1 \pm 0.8$  ( $n=11$ ) mg saliva from the operated gland and  $0.4 \pm 0.2$  ( $n=11$ ) and  $1.9 \pm 0.4$  ( $n=11$ ) mg saliva from the contralateral gland; and finally phenylephrine 30 and 100  $\mu\text{g}/\text{kg}$   $3.8 \pm 0.3$  ( $n=6$ ) and  $10.9 \pm 1.1$  ( $n=6$ ) mg saliva from the operated gland and  $0.2 \pm 0.1$  ( $n=6$ ) and  $2.4 \pm 0.6$  ( $n=6$ ) mg

saliva from the contralateral gland. As to isoprenaline the amount of saliva secreted was at a dose level of 5  $\mu\text{g}/\text{kg}$   $7.1 \pm 0.2$  ( $n=10$ ) from the operated gland and  $1.2 \pm 0.3$  ( $n=10$ ) mg saliva from the contralateral gland and at a dose level of 8  $\mu\text{g}/\text{kg}$   $3.1 \pm 0.3$  ( $n=10$ ) and  $1.9 \pm 0.4$  ( $n=10$ ) mg saliva, respectively.

**Sympathetic denervation + desmethylinipramine.** In those rats where the neuronal pump was inactivated previously to the administration of noradrenaline, adrenaline and phenylephrine a lower threshold dose in the operated than in the contralateral gland could only be demonstrated for noradrenaline; however the difference in threshold between operated and contralateral gland was small: the dose needed to evoke secretion in the operated gland was not even half of that needed to evoke secretion in the contralateral gland. When comparing the threshold doses of the contralateral gland in the desmethylinipramine treated rats with those of the contralateral glands in rats not treated with this drug it can be seen from Table 1 that the threshold doses in the treated rats are lower than those in the untreated rats. In response to submaximal doses of noradrenaline and adrenaline the

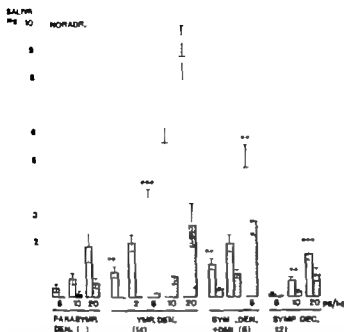


Fig. 2. Noradrenaline. Secretory responses, in mg saliva, of various intravenous doses, in  $\mu\text{g/kg}$ , of noradrenaline from parotid glands, nonoperated (hatched columns) or either peripherally sympathetically denervated or sympathetically denervated or decentralized, 3 weeks in advance (open columns); DM indicates the presence of desmethyldipramine. For further explanations see Fig. 1.

med gland secreted larger amount of saliva than the contralateral gland although the difference was not at all as marked as in the group of rats where these drugs were tested without the presence of desmethyldipramine. For instance, noradrenaline 2 and 5  $\mu\text{g/kg}$  evoked a secretion of  $0.3 \pm 0.1$  ( $n=8$ ) and  $4.8 \pm 0.8$  ( $n=8$ ) mg saliva from operated gland, and from the contralateral gland  $0.2 \pm 0.1$  ( $n=8$ ) and  $1.8 \pm 0.5$  ( $n=8$ ) mg of saliva, and isoprenaline, 5 and 10  $\mu\text{g/kg}$ , evoked secretion of  $0.3 \pm 0.1$  ( $n=9$ ) and  $2.1 \pm 0.2$  ( $n=9$ ) mg saliva from operated gland, and  $1.0 \pm 0.1$  ( $n=9$ ) and  $1.7 \pm 0.2$  ( $n=9$ ) mg saliva from the contralateral gland. In a group of rats neither noradrenaline nor adrenaline was tested at the dose level of 20  $\mu\text{g/kg}$ . In response to phenylephrine 50 and 100  $\mu\text{g/kg}$ , the amount of saliva secreted from the operated gland did not differ significantly from that secreted from the contralateral gland neither were there any differences to be found were the comparisons between operated and contralateral gland to be based on mg saliva secreted per mg dry tissue weight. In the rats treated with desmethyldipramine the contralateral

glands secreted larger amount of saliva than the corresponding glands in the rats not treated with this drug, as can be seen in the Figs. 3-4.

**Sympathetic decentralization** The threshold dose for methacholine in the separated gland did not differ significantly from that in the contralateral gland neither was there any difference to be found for phenylephrine. For noradrenaline and isoprenaline the threshold dose in the operated gland was about half of that in the contralateral gland. For adrenaline the threshold dose was also lower in the operated than in the contralateral gland, although the difference in threshold dose between the glands was not as big as that found for noradrenaline and isoprenaline. In response to submaximal doses of methacholine and phenylephrine a difference between operated and contralateral gland could only be demonstrated at some dose levels provided the amount of saliva secreted was related to the tissue weight. Thus at 5 and 10  $\mu\text{g/kg}$  of methacholine the operated gland secreted  $0.1364 \pm 0.0139$  ( $n=11$ ) and  $0.2683 \pm 0.0213$  ( $n=11$ ) mg saliva per mg gland while the contralateral gland secreted  $0.1099 \pm$

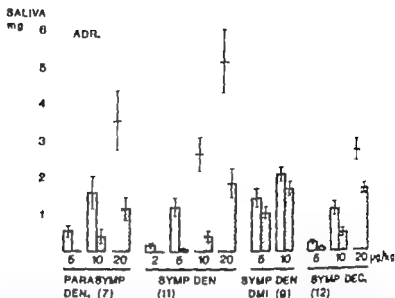


Fig 3 Adrenaline Secretory responses in mg saliva of various intravenous doses, in  $\mu\text{g/kg}$  of noradrenaline from parotid glands unoperated (hatched columns) or either parasympathetically denervated or sympathetically denervated or decentralized 3 weeks in advance (open columns). DMI indicates the presence of desmethylampramine. For further explanations see Fig 1.

0.0105 ( $n=11$ ) and  $0.7797 \pm 0.0193$  ( $n=11$ ) mg saliva per mg gland respectively the difference was significant at a  $p$  level of  $<0.05$ . At 100  $\mu\text{g/kg}$  of phenylephrine the operated gland secreted  $0.1743 \pm 0.0709$  ( $n=12$ ) and the contralateral gland  $0.0904 \pm 0.0179$  ( $n=12$ ) mg saliva per mg gland ( $p<0.07$ ). With regard to submaximal doses of noradrenaline

adrenaline and isoprenaline the amount of  $\alpha$  secreted in response to these drugs was larger from the operated than from the contralateral gland. In instance the operated gland secreted 0.4 ( $n=17$ ) and the contralateral gland  $0.2 \pm 0.1$  ( $n=17$ ) mg saliva after the injection of noradrenaline  $\mu\text{g/kg}$  and after 70  $\mu\text{g/kg}$  of this drug 16

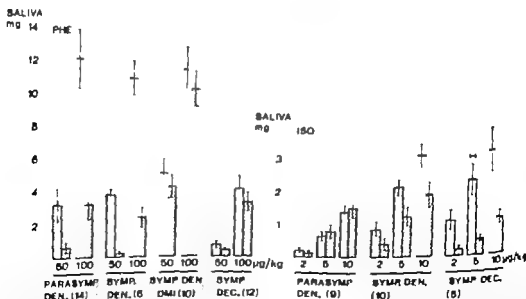


Fig 4 Phenylephrine to the left and isoprenaline to the right. Secretory responses in mg saliva of various intravenous doses, in  $\mu\text{g/kg}$ , of phenylephrine and of isoprenaline from parotid glands unoperated (hatched columns) or parasympathetically denervated or sympathetically denervated or decentralized 3 weeks in advance (open columns). DMI indicates the presence of desmethylampramine. For further explanation see Fig 1.

and  $0.8 \pm 0.2$  ( $n=12$ ) mg saliva, respectively. 10 and 20  $\mu\text{g/kg}$  caused a secretion  $\pm 0.2$  ( $n=12$ ) and  $1.8 \pm 0.3$  ( $n=12$ ) mg saliva from the operated gland and  $0.5 \pm 0.1$  ( $n=12$ ) and 3 ( $n=12$ ) mg saliva from the contralateral gland. In this type of experiment the most marked responses are obtained in response to isoprenaline or response to 5 and 10  $\mu\text{g/kg}$  of the drug. The operated gland secreted  $2.3 \pm 0.4$  ( $n=5$ ) and 6 ( $n=5$ ) mg saliva, and the contralateral gland  $0.5 \pm 0.1$  ( $n=5$ ) and  $1.1 \pm 0.2$  ( $n=5$ ) mg saliva, respectively.

## DISCUSSION

Parotid gland cells were found to be sensitized postsynaptically using the threshold dose method and the amount of saliva secreted in response to supramaximal doses of the drugs as a test for supersensitivity (see Emmelin 1952). The type of operation giving rise to characteristic responses of the auriculo-temporal nerve in the rat is an almost complete disappearance of the sympathetic nerves within the glands judged by the marked reduction in the activity of choline acetyltransferase (Ekström 1974) and in the number of noradrenergic positive nerves (Alm & Ekström 1976). The number of adrenergic nerves does not appear to be reduced by avulsion of the nerve (Alm & Ekström 1977). As would be expected such a sympathetically denervated gland showed a reduced sensitivity towards methacholine in a pilot study on the rat parotid gland (Alm & Ekström 1976). Here the secretory responses to threshold doses of methacholine were tested and supersensitivity to this drug was demonstrated following sympathetic denervation. Supersensitivity is normally considered an unspecific phenomenon (Cannon & Rosenblueth 1949) and in the present study sensitization to some of the sympathomimetic drugs as found in the rat parotid gland in the presence of  $\alpha$ - and  $\beta$ -adrenoceptors both during secretion of fluid has previously been demonstrated (Ekström 1973). In the present investigation the observation was made that following sympathetic denervation it was particularly the  $\beta$ -adrenoceptors of the gland that were sensitized. Some of the marked glandular atrophy the secretory responses to isoprenaline were not even registered in the denervated gland when the

amount of saliva produced was related to the tissue weight. The results obtained in connection with H 80/62, the  $\beta$ -selective adrenoceptor stimulating drug did also vary.

Removal of the superior cervical ganglion causes most of the adrenergic nerves in the rat parotid gland to degenerate (Alm & Ekström 1977); such a denervation does not seem to affect the cholinergic nerves of the gland as judged both from studies using histochemistry (Alm & Ekström 1976) and from those determining the activity of choline acetyltransferase (Ekström 1977). In the sympathetically denervated parotid gland the sensitization developed was particularly marked to noradrenaline, adrenaline and phenylephrine. It was less marked to isoprenaline and even less to methacholine. In the presence of desmethylinpramine the contralateral gland became sensitized which had the consequence that the difference in the response to noradrenaline and adrenaline between the sympathetically denervated gland and this gland was diminished and to phenylephrine a significant difference could no longer be demonstrated. Isoprenaline was not tested in animals given desmethylinpramine since it is not considered to be taken up by the neuronal amine pump (Iversen 1967).

The sensitization developed in the sympathetically decentralized gland to noradrenaline and adrenaline was below that attained after sympathetic denervation. This was however not the case for isoprenaline. As to phenylephrine and also to methacholine increased secretory responses from the decentralized gland could only be obtained at some dose levels provided the responses were expressed per tissue weight. It may thus be concluded that sympathetic denervation of the rat parotid gland is followed by a sensitization that is both of pre and postfunctional type. The latter type not only indicated by the hypersensitivity to isoprenaline but also by the unspecific supersensitivity to methacholine. It was predominantly of the prefunctional type to noradrenaline and adrenaline and probably entirely so to phenylephrine as shown by the use of desmethylinpramine. Further the postfunctional supersensitivity developed towards the sympathomimetic drugs seemed particularly to be mediated via  $\beta$ -adrenoceptors, as seemed to be the case also for the postfunctional supersensitivity developed after the sympathetic decentralization of the gland.

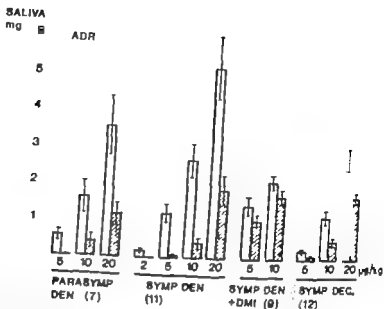


Fig 3 Adrenaline Secretory responses in mg saliva, of various intravenous doses, in  $\mu\text{g/kg}$  of noradrenaline from parotid glands unoperated (hatched columns) or either parasympathetically denervated or sympathetically denervated or decentralized 3 weeks in advance (open columns) DMI indicates the presence of desmethylimipramine. For further explanations see Fig 1

0.0105 ( $n=11$ ) and  $0.2297 \pm 0.0193$  ( $n=11$ ) mg saliva per mg gland respectively the difference was significant at a  $p$  level of  $<0.05$ . At 100  $\mu\text{g/kg}$  of phenylephrine the operated gland secreted  $0.1243 \pm 0.0709$  ( $n=12$ ) and the contralateral gland  $0.0904 \pm 0.0129$  ( $n=12$ ) mg saliva per mg gland ( $p<0.07$ ). With regard to submaximal doses of noradrenaline

adrenaline and isoprenaline the amount of saliva secreted in response to these drugs was larger from the operated than from the contralateral gland. For instance the operated gland secreted  $0.164 \pm 0.014$  ( $n=12$ ) and the contralateral gland  $0.02 \pm 0.01$  mg saliva after the injection of noradrenaline  $\mu\text{g/kg}$  and after 20  $\mu\text{g/kg}$  of this drug

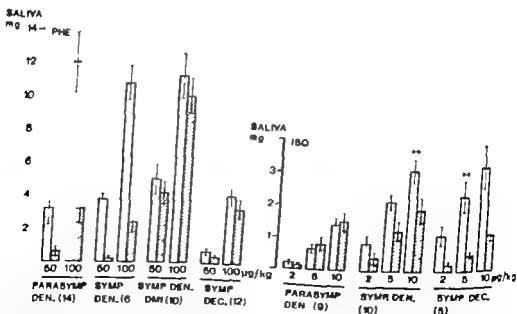


Fig 4 Phenylephrine to the left and isoprenaline to the right. Secretory responses in mg saliva of various intravenous doses, in  $\mu\text{g/kg}$ , of phenylephrine and of isoprenaline from parotid gland unoperated (hatched columns) or either parasympathetically denervated or sympathetically denervated or decentralized 3 weeks in advance (open columns) DMI indicates the presence of desmethylimipramine. For further explanations see Fig. 1

reponderance for an  $\alpha$ -mediated sensitization rat parotid gland following parasympathetic resection.

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The finding that the sympathetically decentralized parotid gland lacked obvious signs of sensitization to methacholine makes it likely that the supersensitivity found towards this drug in rats given a liquid diet (Ekström & Templeton 1977) and in those treated with a ganglion blocking drug (Ekström & Lindmark 1978) was primarily due to reduction or abolishment of the traffic of secretory impulses in the postganglionic parasympathetic nerves of the parotid gland and not to changes in the postganglionic sympathetic nerves of the gland.

Many studies have been devoted to the effect of parasympathetic denervation and decentralization on the sensitivity of salivary glands (see Emmelin 1965). Only few studies have dealt with the effect of sympathetic denervation and decentralization; one reason for this is probably the considerable variation in the sympathetic secretory innervation of salivary glands. However, in the cat, a submaxillary gland Emmelin & Engström (1960) found after sympathetic denervation supersensitivity to noradrenaline and adrenaline but not regularly to parasympathetic drugs. Sympathetic decentralization of this gland caused no supersensitivity which suggested to these authors that the traffic of secretory impulses in the adrenergic nerves of the gland was low. In the rat both submaxillary and parotid glands are supplied with sympathetic nerves that take part in digestive reflexes causing secretion both of fluid and of organic material (Ohlin 1968; Garrett & Harrop 1976; Speirs & Hodgson 1976). Ohlin (1968) found the sympathetically decentralized submaxillary gland to weigh slightly less than its contralateral gland and further it seemed to have a lower threshold dose for noradrenaline than its control. In the present study the sympathetically decentralized parotid glands were also found to weigh less than their contralateral glands and it was possible to demonstrate significant differences in the sensitivity to sympathomimetic drugs between decentralized and control glands.

When comparing the secretory responses towards noradrenaline, adrenaline and isoprenaline from the sympathetically denervated gland in the case of noradrenaline and adrenaline in the presence of desmethylinipramine with those from the sympathetically decentralized gland a striking feature seems to be that the degree of postjunctional sensitization developed after denervation appears to be similar to that after decentralization. A similar relationship between sympathetically denervated

and sympathetically decentralized parotid glands in the rat was recently found in connection with sensitization that was demonstrated towards a selective  $\beta$ -adrenoceptor stimulating drug B K- (Ekström 1979). Parasympathetic denervation of salivary glands is known to cause a more pronounced supersensitivity than parasympathetic decentralization and this is explained by the latter the denervation does not only exclude the action of the gland cells of that fraction of the transmitter which is released by the arrival of secretory impulses but also the action on the gland cells of that fraction of the transmitter that is continuously released from the nerve endings (see Emmelin 1965). The findings of the present study combined with those of Ekström (1979) may suggest the possibility that the continuous release of the adrenergic transmitter of minor importance for the level of secretion of the parotid gland cells; this may depend on an insufficient amount of the transmitter acting on the receptors perhaps due to too a small continuous release of the transmitter from the nerve endings or to an ineffective amine uptake mechanism at the end of the certain continuous release of the adrenergic transmitter in analogy with that of the cholinergic transmitter has previously been discussed by Emmelin (1967) the fact that in the present investigation unambiguous signs of supersensitivity towards methacholine was found after sympathetic denervation but not after sympathetic decentralization may perhaps be taken as a support for this possibility.

In salivary glands parasympathetic and sympathetic nerves are considered to act on the same secretory cell (see Emmelin 1967). Supersensitivity was mainly the  $\alpha$ -adrenoceptors that in the present study seemed to mediate sensitization to sympathomimetics following parasympathetic denervation. This may imply that in the rat parotid gland not all cells supplied with  $\beta$ -adrenoceptors are under the influence of the parasympathetic nerves. On the other hand it has been suggested from *in vitro* studies on slices on rat parotid tissue that the same cell is supplied both with  $\alpha$ - and  $\beta$ -adrenoceptors and with muscarinic cholinergic receptors further that  $\alpha$ -adrenoceptors and cholinergic receptors use the same intracellular mechanism whereas  $\beta$ -adrenoceptors use another one (Schramm & Linger 1975; Leslie, Putney Jr & Sherman 1977). It is tempting to hypothesize that such an intracellular arrangement may be the action for the fast

# anaerobic threshold skeletal muscle enzymes and fiber composition in young female cross-country skiers

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Anaerobic threshold (AT) and maximum oxygen uptake ( $\text{max } V_{O_2}$ ) were determined in 15 young female cross-country skiers, aged 15-20 years, during incremental bicycle ergometer exercise. Succinate dehydrogenase (SDH), malate dehydrogenase (MDH), citrate synthase (CS) and lactate dehydrogenase (LDH) were analyzed biochemically and percentage of slow twitch fibres (RST fibres, exposed adenine triphosphatase staining) histochemically in muscle samples obtained from m. vastus lateralis.  $\text{Max } V_{O_2}$  correlated significantly with anaerobic threshold in  $\text{ml kg}^{-1} \text{ min}^{-1}$  (mlAT) but when AT was expressed in percent of  $\text{max } V_{O_2}$  (rAT) the correlation was insignificant. Significant correlations were found between rAT and SDH ( $r=0.63$ ) and between mlAT and CS ( $r=0.58$ ).  $\text{Max } V_{O_2}$  showed no significant correlations with the enzymes studied or RST fibres. The results of the study seem to support the hypothesis that anaerobic threshold is related to oxidative capacity of muscle.

**Key words:** Anaerobic threshold, maximum oxygen uptake, enzyme activities, muscle fiber composition, athletes.

It is well established that endurance training will induce significant functional and structural adaptations both in the cardiovascular system and in skeletal muscle. Maximum oxygen uptake ( $\text{max } V_{O_2}$ ) is thought to reflect these adaptations because it has been shown to correlate with heart rate, cardiac output, muscle capillarization, muscle fiber composition, mitochondrial density, activities of oxidative enzymes etc. (see Holloszy 1976, Booth 1976, Saltin 1977, Astrand & Rodahl 1977).  $\text{Max } V_{O_2}$  might be the most important determinant of endurance performance when large muscle mass is activated during maximum exercise of short duration. During prolonged exercise lasting 3 h, 60-90% of  $\text{max } V_{O_2}$  can be used. It has been suggested that the endurance of athletes can be characterized physiologically as the  $\text{max } V_{O_2}$  as the highest intensity of exercise at which the production of lactic acid is not increased (Hindmarsh 1969, Cozill et al 1973). Incremental bicycle ergometer tests have been

developed to estimate this intensity of exercise that is termed the anaerobic threshold (AT). AT may be detected from the increase in blood lactate concentration above normal resting levels and the non-linear increase in minute ventilation and carbon dioxide production (Wasserman et al 1973, Davis et al 1976, Mader et al 1976, Bachl et al 1978).

The purpose of this study was to determine the anaerobic threshold of young female cross-country skiers and to investigate the relationships between anaerobic threshold, maximum oxygen uptake and oxidative capacity of skeletal muscle.

## MATERIAL AND METHODS

The subjects were 15 female cross-country skiers, aged 15 to 20 years. They were among the best girl skiers in Finland in their age groups and had trained (running,

We deeply regret that our honoured and dearly beloved teacher Professor Eino Karvonen, Ph.D. died before the publication of this paper.





2.1. *Maximal and anaerobic threshold—mean values of the 15 subjects*

Max $\dot{V}_{O_2}$ (l $\times$ min <sup>-1</sup> )	STPD (ml $\times$ kg <sup>-1</sup> min <sup>-1</sup> )	Max heart rate	Max blood lactate	Anaerobic threshold		
				(ml $\times$ kg <sup>-1</sup> min <sup>-1</sup> )	%max $\dot{V}_{O_2}$	Heart rate
69	47.3	193.5	8.2	40.9	85.7	180.4
6.22	3.6	8.1	1.4	3.3	8.6	9.2

ness value of %ST fibres and muscle fibres of SDH, MDH, CS and LDH are presented in Table 3. On the average the girl skiers had 68% ST fibres in their m. vastus lateralis. The fibres showed no significant correlation with  $\dot{V}_{O_2}$  max or max  $\dot{V}$ . SDH correlated significantly with %AT ( $r = -0.63$ ,  $P < 0.05$ ) and CS with  $\dot{V}$  ( $r = 0.58$ ,  $P < 0.05$ ). Sum of the z-scores of the three enzymes (%OE) correlated significantly with %AT ( $r = 0.54$ ,  $P < 0.05$ ). The correlations between max  $\dot{V}_{O_2}$  and the enzymes studied were significant. LDH correlated significantly with intensity of training ( $r = -0.51$ ,  $P < 0.05$ ) and with the subjects ( $r = -0.51$ ,  $P < 0.05$ ).

## DISCUSSION

The concept of anaerobic threshold has been used to describe the intensity of exercise or at consumption at which a person can work prolonged time. It may be detected from the rise in blood lactate concentration and associated changes in respiratory parameters during maximal exercise (Wasserman et al 1973). Changes have been thought to reflect the onset of metabolic acidosis and to show that the rate of the aerobic energy production is no more sufficient to fulfill the requirements of exercise. In our study the intensity of exercise was increased every second minute by 15 W corresponding to an increase of 0.15–0.20 l  $\times$  min<sup>-1</sup> in  $\dot{V}_{O_2}$ . It was supposed that the individual AT values lay between two successive work loads, therefore it was tried to determine the AT at the end of 5 W or 0.05–0.10 l  $\times$  min<sup>-1</sup> (1 ml  $\times$  min<sup>-1</sup>). This corresponds to an increase of 10–15% in  $\dot{V}_{O_2}$ . In some cases %AT was surprisingly high. For instance subject 2 attained an AT value of 45 ml  $\times$  kg<sup>-1</sup>  $\times$  min<sup>-1</sup> which is 3rd in the group. After that intensity of exercise increased and blood lactate concentra-

tion increased from 4.1 mM to 8.2 mM. Apparently she was not able to increase her  $\dot{V}_{O_2}$  after she had attained the AT. Consequently her max  $\dot{V}_{O_2}$  was relatively low and %AT value clearly highest in the group. Four months later the %AT of this girl was still highest in the group.

In our study a 4 mM (Mader et al 1976) threshold value of blood lactate concentration was applied. It has been shown that after marathon race the blood lactate concentration of runners is 1.1–3.5 mM (Costill & Fox 1969) and according to Costill et al (1973) athletes are able to work for prolonged time at blood lactate level of 4 mM or lower if the intensity of exercise is higher the blood lactate concentration increases all the time. In our study some of the subjects attained high blood lactate values already at low intensity of exercise. For instance subject 12 had blood lactate concentration of 4.3 and 4.8 mM after the 5th and 7th work loads respectively (see Fig. 1). The beginning intensity of exercise and the first 30 W increments might have been too high in relation to the working time of two minutes at each intensity of exercise. An oxygen deficit could have been developed and as a result lactate production starts to increase. On the other hand some of the subjects had blood lactate values below 2 mM at low intensity of exercise indicating that oxygen deficit had not been high in the beginning of the test. For instance

Table 3. *Muscle fiber distribution and activities of the enzymes studied*

n=15	% ST fibres	Enzyme activities, (nmol min <sup>-1</sup> $\times$ mg <sup>-1</sup> prot)			
		SDH	MDH	CS	LDH
$\bar{x}$	59.9	13.9	1.776	144	839
S.D.	9.6	2.8	469	41	182

Table 1 Characteristics and training data for all subjects

<i>n</i> = 15	Age (y)	Height (cm)	Weight (kg)	Body fat <sup>a</sup> (%)	Quantity of training during the preceding year <sup>b</sup> (km × year <sup>-1</sup> )
$\bar{x}$	17.6	166.9	57.7	22.7	3 330
S.D.	1.4	5.1	5.1	2.3	670

<sup>a</sup> Skinfold estimation according to Durnin & Rahaman (1967)

<sup>b</sup> Obtained from the training diaries of the subjects

walking, skiing, rollskiing) on the average 3 300 km during the preceding year. Information on their training and their physical characteristics are seen in Table 1. The subjects gave their informed consent to participate in the measurements of this study.

AT and max  $\dot{V}_{O_2}$  were determined during performance on a bicycle ergometer (60 rpm). The beginning intensity of exercise was 90 W and the power was increased every second minute by 30 W during the first 2–4 increments (according to the fitness level of the subject) and thereafter by 15 W every second minute until exhaustion which occurred after about 20–25 min of exercise.

During the test ventilation ( $\dot{V}_A$ ), oxygen uptake ( $\dot{V}_{O_2}$ ) and carbon dioxide production ( $\dot{V}_{CO_2}$ ) were measured using a semiautomated system. The subject breathed through a modified Otis McKerrow respiratory valve and the expirations were directed into a smooth tube (Ø 30 mm). The expiratory volume was measured using a heated Fleisch 3 pneumotachograph and a Penyer & Gut flow meter. This system was calibrated before and after the tests by pumping 100 l of air through the pneumotachograph using a 4 l cylinder. Samples from every expiration were collected in relation to flow into a sample bag. For every 30 s period ventilation was recorded and  $O_2$  and  $CO_2$  concentrations were analyzed from a sample bag calibration gas (analyzed by Scholander technique) and room air by Beckman OM 11 and Godart Capnograph respectively. The temperature of the expired air was measured in the pneumotachograph and the respiratory volumes ( $\dot{V}_A$ ,  $\dot{V}_{O_2}$ ,  $\dot{V}_{CO_2}$ ) were calculated for every 30 s period and corrected for temperature and ambient pressure. The highest sum of two successive 30 s determinations was taken as maximum oxygen uptake.

EKG for heart rate calculations was recorded at the end of every minute. Blood samples for lactate determination (enzymatic method Biochemica Boehringer) were drawn from fingertip after the 5th and the 7th work load and again immediately after and 2.5 min after the test.

To determine AT,  $\dot{V}_A$  and  $\dot{V}_{O_2}$  were plotted against corresponding  $\dot{V}_{O_2}$  and heart rate values (Fig. 1). Departure from linearity in the  $\dot{V}_A$  and  $\dot{V}_{O_2}$ -responses was used as a criterion of anaerobic threshold. The increase of blood lactate concentration over 4 mM was used together with the respiratory responses to verify that AT had been reached. AT was determined by hand and calculated in ml O<sub>2</sub> consumed × kg<sup>-1</sup> × min<sup>-1</sup> (mlAT) and in percent of maximum oxygen uptake (%AT). Correlation between the determinations of two investigators was 0.9. The reliability of the testing procedure was studied separately

on 12 physical education students. The correlation between their test-retest (one week between retests) values were 0.91 (1 × min<sup>-1</sup> and 0.94 ml × kg<sup>-1</sup> × min<sup>-1</sup>) for max  $\dot{V}_{O_2}$  and 0.95 (1 × min<sup>-1</sup>, 80 W × kg<sup>-1</sup> × min<sup>-1</sup>) and 0.80 (% of max  $\dot{V}_{O_2}$ ) for AT.

A muscle sample from m. vastus lateralis of left leg was obtained using needle biopsy technique (Berglund 1962). The specimen was divided into two parts. One portion (10–15 mg) for histochemical analysis was covered with OCT compound and frozen in isopentane cooled in liquid nitrogen. The sample was sectioned in a cryostat and stained for myosin ATPase (Padykula & Hux 1955). Microphotographic enlargements of the slides were used to classify the muscle fibres to slow twitch (ST) and fast twitch (FT) types (Gollnick et al. 1972). The other portion (5–15 mg) of the muscle sample was frozen in liquid nitrogen and stored at -80°C for subsequent enzyme analysis. After smelting on ice-bath these samples were weighed and homogenized in 1.50 ml 0.1 M Tris buffer pH adjusted to 7.5 in an all glass Potter Elvehjem homogenizer operated manually on ice-bath. The following enzymatic activities were assayed from the original homogenate or after appropriate dilutions: succinate dehydrogenase (SDH, E.C. 1.3.99.1 according to Pennington (1961), citrate synthase (CS, E.C. 4.1.3.7) according to Srere (1969), malate dehydrogenase (MDH, E.C. 1.1.1.37) according to Ochoa (1974), lactate dehydrogenase (LDH, E.C. 1.1.1.27) according to Kornberg (1955). All preparation steps were performed at 0–4°C. The specific enzyme activities at 22°C were referred to protein content estimated according to Lowry et al. (1951). To estimate the total oxidative capacity of the muscle the sum of the activities of the three oxidative enzymes (ΣOE) was calculated.

## RESULTS

Mean max  $\dot{V}_{O_2}$  of the subjects was 2.69 l min<sup>-1</sup> or 47.3 ml × kg<sup>-1</sup> × min<sup>-1</sup>. The AT averaged 40.9 ml × kg<sup>-1</sup> × min<sup>-1</sup> which was 86% of max  $\dot{V}_{O_2}$  (Table 2). Maximal oxygen uptake (ml × kg<sup>-1</sup> × min<sup>-1</sup>) correlated positively ( $r = 0.60$ ,  $P < 0.01$ ) with mlAT but correlation between max  $\dot{V}_{O_2}$  and %AT was insignificant. The age of the subjects correlated positively with %AT ( $r = 0.54$ ,  $P < 0.01$ ) and negatively with max

63  $P < 0.05$ .

er and blood and (3) oxygen transport to the muscles. In our study, high quantity of lactate seemed to be related to low LDH activity and anaerobic threshold. Although the negative correlation between LDH and %AT ( $r = -0.4$ ) is insignificant, these results seem to show clearly that the ability to keep the blood lactate concentration at low level might also be related to capacity of the muscle to produce lactate. The highest measured max  $\dot{V}_{O_2}$  values have been found from cross-country skiers (Åstrand & Rodahl 1977). In our study the mean max  $\dot{V}_{O_2}$  was  $3.7 \text{ ml} \times \text{kg}^{-1} \times \text{min}^{-1}$ . Higher values for females of the same age and for cross-country skiers have been reported (Costill et al. 1976; Åstrand & Rodahl 1977; Rusko et al. 1978). To explain the low values observed in this study we can be explained by methodological differences. During bicycle ergometer exercise the max  $\dot{V}_{O_2}$  was 7% lower than during treadmill running. In a study the duration of bicycle ergometer test was 20–25 min which is not optimal for attaining max  $\dot{V}_{O_2}$  (Åstrand & Rodahl 1977). The tests performed 3 months after the main competition and according to Simon et al. (1978) cross-country skiers have lowest max  $\dot{V}_{O_2}$  values at the end of the competition season. The results of the study allow to present a correlation of the effect of training in young female cross-country skiers. The significant correlation between %AT and training ( $r = 0.70$ ,  $P < 0.01$ ) indicates that as the skiers become older they train quantitatively more. The age of the subjects correlated positively with %AT while negative correlation between age and max  $\dot{V}_{O_2}$  was observed. Accordingly increased amount of training of the skiers seems to be influenced more on anaerobic threshold than on maximum oxygen uptake. This result is in agreement with our observations concerning training of Finnish cross-country skiers.

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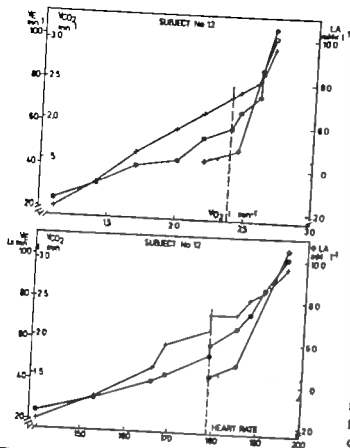


Fig. 1 Example of anaerobic threshold (AT) determination. To determine AT  $\dot{V}\text{CO}_2$  and blood lactate values were plotted against corresponding  $\dot{V}\text{O}_2$  (upper curves) and heart rate (lower curves) values. Dashed vertical line denotes AT.

the blood lactate concentrations of subject 1 were 0.8 and 1.5 mM after the 5th and 7th work loads. Because the maximum blood lactate concentration of subject 1 was 5.0 mM (4 months later 5.6 mM) and that of subject 12 0.4 mM, the overall capacity of the body to produce energy anaerobically could explain the observed differences in blood lactate levels during submaximum exercise. Accordingly, the abrupt increase in blood lactate concentration together with the respiratory responses might be more feasible than a rigid 4 mM threshold as a criterion of anaerobic threshold.

It has been proposed that during prolonged submaximal exercise the most important determinant of performance might be the oxidative capacity of the muscles (e.g. Holloszy 1967, Costill et al 1976, Fink et al 1977, Rusko et al 1978). In this study the relationships between anaerobic threshold, maximum oxygen uptake and selected variables of oxidative capacity of muscles were investigated. The high activities of oxidative enzymes could

increase the capacity of the muscles to oxidize pyruvate during submaximum exercise when the oxygen supply to the working muscles is probably adequate. Consequently the release of pyruvate to lactate might be decreased and the increases in muscle and blood lactate concentrations occur at higher intensity of exercise than the anaerobic threshold is at higher level. For the enzymes studied SDH and CS activities and the  $\Sigma\text{OE}$  correlated significantly with anaerobic threshold while  $\text{max } \dot{V}\text{O}_2$  showed no significant correlations with the enzyme activities.

The results seem to support the hypothesis that the submaximum or prolonged work capacity expressed as anaerobic threshold might be related to the oxidative capacity of the muscles. During maximum exercise the supply of oxygen to the working muscles may be inadequate. The insignificant correlations between  $\text{max } \dot{V}\text{O}_2$  and the studied enzymes seem to show that early fatigue might more likely be related to central or local factors of circulation than to the oxidative capacity of the muscles (See Clausen 1977, Salin 1978, Rusko et al 1978).

In previous studies significant correlations between  $\text{max } \dot{V}\text{O}_2$  and oxidative enzyme activities in muscle fiber composition have been observed (Costill et al 1976, Rusko et al 1978, Bergh et al 1978). These correlations might have been restricted from the heterogeneity of the subjects. In our study the subjects were all endurance athletes and corresponding correlations were not observed. The different deterioration rates in  $\text{max } \dot{V}\text{O}_2$  and enzyme activities during detraining (Heard et al 1976, Reltman 1976, Ölander et al 1977) may partly explain the lack of correlations between the variables in our group which was measured 4 months after intensive training and competition season. Moreover, the bicycle ergometer exercise of the muscle studied (m VL) are not necessarily the best means to characterize the effects of training in cross-country skiers.

According to our hypothesis a high correlation should exist between anaerobic threshold and oxidative potential of the muscles. Other factors in addition to oxidative capacity of the muscles might also determine the level of anaerobic threshold, e.g. (1) rate of lactate transport from exercising muscles to the blood and the removal of lactate from the blood by heart liver and nonexercising muscles.

# Locomotion of the low spinal cat: coordination within a hindlimb

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Cats were subjected to a complete transection of the spinal cord (T<sub>10</sub>-T<sub>12</sub>) 1-2 weeks after birth. A few days after the operation they could perform alternating limb movements and somewhat later walking movements with their hindlimbs on a treadmill. The stepcycle of the hindlimbs could be divided into a flexion phase (F) and a first (E<sub>1</sub>) second (E<sub>2</sub>) and third (E<sub>3</sub>) extension phase. The duration of the support phase decreased markedly with treadmill velocity while the swing phase decreased to much smaller extent. The pattern of electromyographical activity in hip, knee, ankle and toe muscles during treadmill locomotion was very similar to that of the intact cat. This related to both the timing and the general shape of locomotor bursts. The extensor muscles were thus activated well before the placement of the foot and able to produce enough force to support the body. The propulsive thrust in each step was, however, decreased and the animals showed more severe deficits particularly in their equilibrium control. It is concluded, however, that neural networks in the spinal cord (with its peripheral inflow intact but without supraspinal influences) have the capacity to generate a specific and detailed locomotor pattern.

**Key words:** Spinal cord, spinal transection, locomotion.

Neural control of locomotion can be divided into conceptually different tasks, (i) to generate stereotypic locomotor movements that result in propulsion typical for a given species, (ii) to adapt these movements to the environment and the needs of the animal and (iii) to maintain the equilibrium of the animal during the different body positions encountered during locomotion. The aim of the present study is to investigate the extent to which the cat spinal cord can reproduce stereotypic locomotion (i). It is evident that the different types of anticipatory control subordinated to the movement repertoire of the intact animal, and furthermore that the equilibrium control must be drastically reduced (Eckhorn & Nieuwenhuis (1874), Freusberg (1874) and Jägerskiöld & Freusberg (1874) described that dogs with a low cord transection in the lower thoracic region could not only support themselves but also produce rhythmic sequences of flexion and extension of the hindlimbs constituting reflex steps. The error could be induced by various stimuli but did not occur spontaneously if the animal was held

with the limbs extended. In 1905 Philippon analyzed films obtained from walking spinal dogs and defined the different flexion and extension phases, found in normal animals (see below). The similarity of spinal stepping to normal walking was, however, questioned by many investigators (Magnus 1924, ten Cate 1940). Using different training or manipulation procedures later investigators have been able to improve the walking movements in different kinds of chronic spinal animals (Kellog et al. 1946, Sherringer & Dykman 1951, ten Cate 1964, 1964 and Steltzner et al. 1975). The reports are, however, formulated as rather general descriptions with no effort to define how complete a walking pattern the spinal cord can generate. Noradrenergic precursors or receptor stimulators may also release spinal stepping or walking on a treadmill in acute spinal animals (Grillner 1969, Budakova 1973, Forsberg & Grillner 1973). In this context it is relevant to note that an interneuronal network in the cat's spinal cord has been described which,

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without afferent feedback, can generate a complex pattern of flexor and extensor activity (T. G. Brown 1911, 1914; Jankowska et al 1967a, b; Grillner & Zangger 1978; Vidal et al 1979). Peripheral feedback acting on this network has also been reported (Grillner & Rossignol 1978; Andersson et al 1978).

In comparing the walking movements of spinal and decerebrate cats, Sherrington (1910) found large differences, especially during the "extensor phase" of the step. He believed that a mechanism essential to produce "real" walking was situated "between the levels of anterior colliculus and hind edge of pons".

In this study the locomotion of chronic spinal kittens walking on a treadmill is analyzed, i.e. by recordings of the movements and of the electromyographical activity in the different hindlimb muscles. The specific pattern of the spinal locomotion is then compared to data from walking in the intact cat (e.g. Engberg 1964; Engberg & Lundberg 1969; Goslow et al 1973; Rasmussen et al 1978) or recordings obtained from intact cats walking on the same treadmill. In a first study the characteristics and the timing of the angular movements and muscular activity of one hindlimb are investigated. In a second, the coordination between the two hindlimbs is reported. Some of the results have been mentioned previously (Forsberg, Grillner & Sjöström in Grillner 1973).

## METHODS

**General procedures.** Fourteen kittens from seven litters were subjected to a transection of the spinal cord at a low thoracic level from the 6th to the 17th day after birth. The spinal cord was exposed by a laminectomy; the dura was opened and half a segment of the cord was removed; whereafter the dura was sutured again. The spinal cord was dissected out after the animal was sacrificed and the level and the completeness of the lesions was checked. In all kittens there was a complete lesion between Th10-Th1.

The kittens were brought back to their mothers some hours after the operation and were then nursed and fed by her. The increase of the body weight was followed each day as an indicator of the general health of the kittens. While the mothers usually succeeded in emptying the bladder of the kittens by licking around the perineum, the bladder of each kitten was checked twice daily and manually emptied when needed. Infections usually from the urinary tracts were treated by benzylpenicillin or ampicillin. The kittens were "trained" daily 10 to 20 min on a treadmill.

**Recording procedures.** EMG (electromyography)-elec-

trodes and LEDs (light emitting diodes) for movement recording (see below) were fixed to the kitten in brief Halothane anesthesia. During the experiments the fore part of the kittens were either carried by one of the investigator or were standing with the kitten on a platform. The hindlimbs were on a treadmill belt, which was motor-driven and could be moved at various speeds. The tail was gently held with the other belt to assist the kitten to maintain equilibrium.

**EMG recordings.** Pairs of thin copper wires (100  $\mu$ m insulated except for 1 mm at the tip) were inserted into the desired muscles with hypodermic needles (Griffith & Lundberg 1969; Forsberg & Grillner 1973). The composition was checked by stimulation of each electrode against the ground electrode, which was subcutaneously placed at the back, and simultaneous palpation of the contracting muscle. The signals were pre-amplified (xP15) and high pass filtered (5th order, 300 Hz) to eliminate movement artefacts. They could be displayed on an inkwriter and on a tape recorder, rectified and low pass filtered (5th order Bevel, 100 Hz) and fed to a minicomputer (see below).

**Movement recordings.** The movements were recorded by a Selspot system (Selective Spot Recognition, Sirelcom AB, Partille, Sweden), with a sampling rate of 100 Hz but for our purpose reduced to 16 Hz. Five LEDs (light emitting diodes) were glued to the distal limb at the pelvis (iliac crest), hip, knee, ankle and at tarsometatarsal joint. The light pulse from each diode detected by a special optoelectronic detector outside the focal plane of a camera and the position was stored with processing electronics. The x and y coordinates available in digital form, to be fed into the computer for some LEDs in an analogue form. Attention was paid to avoid reflexions in the floor and walls from the LEDs and other infra red radiations.

**Recordings of force reactions.** The reaction force was ground to the foot during the support phase was measured on a piezoelectric force plate (Kistler model 913). The kitten was held in a similar way as when it walked on the treadmill but stepped instead on the force plate. The vertical force ( $F_z$ ) and the horizontal forces in the longitudinal ( $F_y$ ) and transverse ( $F_x$ ) directions were recorded on the inkwriter and the tape-recorder and fed into the computer. Before entrance to the computer the signals were low pass filtered (5th order Bevel, 100 Hz).

**Storage of data.** The low pass filtered signals of muscular activity and of the force reactions were converted to digitally converted with a sampling rate of 31 Hz. Special hardware was developed to control the conversion and synchronize it to the Selspot system. All the data were stored on a minicomputer (HP 21MX) and temporarily on disc. In this way the data could be compared obtained during 90 s. Directly after each recording (normally 10 to 30 s) the data were stored on a magnetic tape and new recordings could start.

The raw (unfiltered and not rectified) EMG analogue signal from the force plate and usually a analogue signals of the x and/or y coordinate (if LED glued to the toe could be recorded on an LED kymograph frequency response 50 Hz) and of

to extend during the end of  $E_1$ . After foot contact the hip continued to extend while the knee and ankle yielded (flexed) under the load ( $E_2$ ) to partly extend again ( $E_3$ ) (see Fig. 1C and Legend). During slow walk the extension in the hip and ankle at the end of the support phase was weak (see Fig. 1C and Fig. 4A) which made it difficult to determine the transition from  $E_2$  to  $E_3$  and, therefore, these two phases have been put together in the plots. Knee and ankle thus closely linked throughout the stepcycle. In the hip proceeds in monotonic flexion-extension movements in phase with knee and ankle movements in phase during  $E_1$ - $E_3$ .

#### Adjustment in the stepcycle

The velocity of the backward movement of the limb during the support phase was controlled by the speed of the belt. During faster walks the duration of the stepcycle shortened, from 880 ms at 0.14 m/s to the half (450 ms) at 0.70 m/s (see Fig. 1A). The reduction was mainly due to a shortening of the support phase from 570 ms to 190 ms whereas the forward swing was reduced to a much smaller extent (with 60 ms, see Fig. 1A). When the swing phase was subdivided into F and E-F was found to decrease only slightly (160→190 ms) but E-F somewhat more (190→100 ms). In relative terms the fraction of the stepcycle taken up by  $E_1 + E_2$  (i.e. the support phase) will decrease with speed and F will increase (Fig. 2B).

The amplitude of the angular movements of the hip was especially those of the knee and ankle, increased at faster speeds (Fig. 2C). The amplitude of the rotational movement of the foot during the support phase increased about 66% from 0.07 m/s to 0.70 m/s in one kitten. The lengthening was mainly due to the increased joint movements of the limb at the ankle to a much less degree to movements of the hip. The ankle had a tendency to hyperextend in the flexion (see Fig. 1C). This was probably due to the passive torque at the end of the support (see Legend) and a weakness of the flexor muscles.

The activity in the extensor muscles shortened at faster speeds, in parallel with the support phase from 700 ms to 300 ms (see Fig. 1D). In contrast, the duration of the knee flexor activity increased at the slowest speeds to remain approximately constant (60-140 ms) (see Fig. 1D) of the support phase in Fig. 1A). The total amount of muscle activity was approximated by integration of the

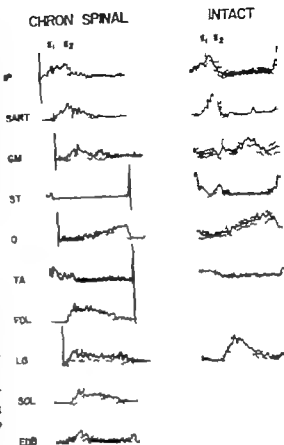


Fig. 3. Averaged EMG from spinal and intact cats. The rectified and filtered EMG from the muscles indicated are averaged after normalization of the stepcycle duration. The mean is indicated as a continuous line and the S.D. as dotted lines. The recordings are from different cats (chron. spinal = 2; intact = 2) and from different experimental sessions but they still be comparable since the cycle durations are chosen to be around 800 ms ( $\pm 20$  ms) and the onset of the stepcycle is triggered by the movement in the ankle where the onset of the flexion and the first extension phases have been used as trigger points. The different phases are marked as lines. The number of averaged locomotor bursts vary between 8 and 12. The position of the FDL electrode (see abbrev.) was confirmed by the forceful digit flexions elicited by each stimulus (see Method).

rectified and filtered EMGs (see Methods). In the knee flexor ST it increased steadily as the kitten walked faster (see Fig. 2E). At lower speeds this was mainly due to the increase of the duration (see Fig. 2D) but at higher speeds to the increased intensity (see Fig. 2F). In the extensor muscle Q the activity decreased to 40% at faster speeds (Fig. 2E). This was not only due to the marked reduction

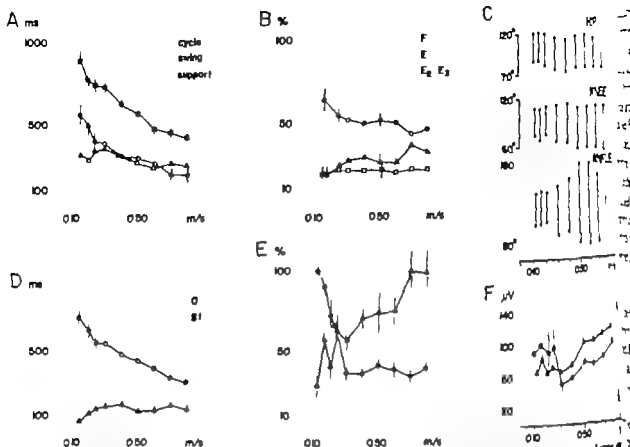


Fig. 2 Adjustments in the stepcycle with speed. The data points are collected from one spinal kitten during an experimental session during different speeds of walk but are representative for the majority of the kittens. In A the mean and S.D. (exceeding 20 ms) of the durations of the stepcycle of the swing phase and of the support phase are plotted. Symbols indicated in A, B and D (D is also applicable to E and F). In B the mean and S.D. (exceeding 5%) of the first phase, the first extension phase and of the sum of the second and third extension phases are represented as percentage total stepcycle duration at different speed. In C the extremes of the angular movements are shown for each speed. D shows the durations of the locomotor bursts of the extensor (Q) and the flexor (St) of the knee (mean  $\pm$  S.D., exceeding 20 ms) and E the total amount of the electrical activity during the locomotor bursts calculated by integration of the rectified and filtered EMGs (mean  $\pm$  S.D.). In order to compare the two muscles in the same plot the maximal value of mV s for each muscle is defined to 100%. In F the means and S.D.s for the mean intensity of the locomotor bursts are plotted. The numbers of observation vary from 8 to 111 in each data point.

trunk, so that both hindlimbs pointed towards one side. As the kittens grew older they would support themselves with the forelimbs held vertically as ordinary cats do. The hindlimbs were kept as above or could occasionally be used to support the animal for several consecutive steps before it fell to one side or the other. If however the hindquarters were prevented from falling by gently holding the tail the kittens could support their body weight and the hindlimbs participated in walking. Placed on a treadmill the kittens could adjust to the belt speed (cf. Forsberg & Grillner 1973; Budakova 1973). Young kittens could track the treadmill over a rather wide range of velocities but it has been our impression that the older preparations would produce "good walking" only in a more narrow speed

range. All kittens could walk, i.e. perform alternating hindlimb movements (as in walk or trot), and some of them would switch over to a more simultaneous activation of the limbs as in gallop at higher treadmill speeds. The coordination between the two hindlimbs will be dealt with separately (Forsberg et al. 1980).

**The structure of the step cycle.** During treadmill locomotion (see Methods) the foot was placed on the belt and the limb then moved backwards in relation to the body during the support phase.

The swing phase started with a simultaneous flexion phase (F) in the hip, knee and ankle and continued with an extension of the knee and ankle in the 1st extension phase ( $E_1$ ) while the hip continued to flex (see Fig. 1, cf. 4 A and B). The

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stepcycle shortened, from 880 ms at 0.14 m/s  
at the half (430 ms) at 0.70 m/s (see Fig. A)  
flexion was mainly due to a shortening of  
support phase from 570 ms to 190 ms whereas  
ward swing was reduced to much smaller  
with 60 ms, see Fig. A). When the swing  
was subdivided into F and  $E_1$ , F was found to  
be only slightly (160→150 ms) but  $E_1$  some-  
more (150→100 ms). In relative terms the  
fraction of the stepcycle taken up by  $E_1$ + $E_2$  (i.e.  
support phase) will decrease with speed and F  
be (Fig. 2B).

amplitude of the angular movements of the  
especially those of the knee and ankle in-  
creased at faster speeds (Fig. 2C). The amplitude of  
medial movement of the foot during the  
support phase increased about 66% from 0.07 m/s  
to 0.14 m/s in one kitten. The lengthening was main-  
ly due to the increased joint movements of the limb  
as a much less degree to movements of the  
The ankle had a tendency to hyperextend in  
flexion (see Fig. C). This was probably due  
to passive torque at the end of the support (see  
flexion) and a weakness of the flexor muscles.

activity in the extensor muscles shortened at  
faster speeds, in parallel with the support phase  
from 700 ms to 300 ms (see Fig. 2D). In contrast,  
duration of the knee flexor activity increased  
slightly at the lower speeds to remain ap-  
proximately constant (60-140 ms, see Fig. D) of  
activity in Fig. A). The total amount of muscu-  
larity as approximated by integration of the

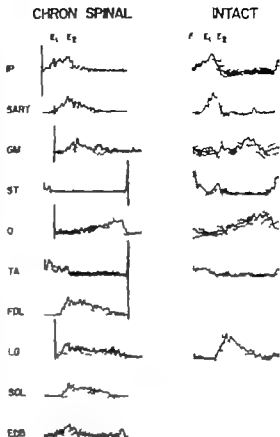


Fig. 3. Averaged EMG from spinal and intact cats. The rectified and filtered EMG from the muscles indicated are averaged after normalization of the stepcycle duration. The mean is indicated as continuous line and the S.D.s as dotted lines. The recordings are from different cats (chron. span = intact = 2) and from different experimental sessions but may still be comparable since the cycle durations are chosen to be around 800 ms ( $\pm 20$  ms) and the onset of the stepcycle is triggered by the movement at the ankle where the onset of the flexion and the first extension phases have been used as trigger points. The different phases are marked as lines. The number of averaged locomotor bursts vary between 8 and 12. The position of the FDL electrode (see abbrev.) was confirmed by the forceful slight flexions elicited by each stimulus (see Methods).

rectified and filtered EMGs (see Methods). In the knee flexor St it increased steadily as the kitten walked faster (see Fig. 2E). At lower speeds this was mainly due to the increase of the duration (see Fig. 2D) but at higher speeds to the increased intensity (see Fig. F). In the extensor muscle Q the activity decreased to 40% at faster speeds (Fig. E). This was not only due to the marked reduction

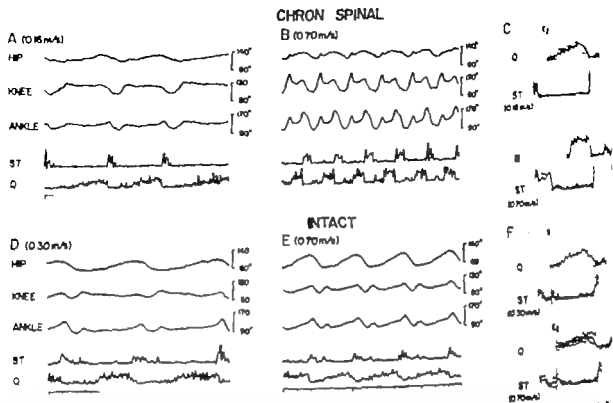


Fig. 4. Comparisons of the changes in locomotor activity at different speeds in chronic spinal and intact cats. A, B, E show the angular movements of the hip, knee and ankle and the EMG's from the flexor (ST) and the extensor (Q) knee during slow (A, D) and fast walk (B, E) from a chronic spinal and an intact cat respectively. In C and F the ST and filtered EMG's are averaged from the actual sessions ( $n=8-12$ ). Q is triggered by the beginning of  $E_1$  and S onset of F. Note that the early Q burst is fully represented (i.e.  $\pm$  S.D.) only after the main Q burst (C and F). The velocity of the belt is indicated for each recording.

of duration with speed (Fig. 2D) but also to a fall of the mean activity in the middle range (Fig. 2F). The mean intensity level at different velocities showed in fact two different slopes. At low speeds it was kept at a constant high level but at 0.30 m/s it suddenly fell to slowly increase again at higher speeds (Fig. 2F). This change occurred at the same approximate speed as the changes in the pattern of the Q-burst (see below and Fig. 4A-C) but a similar tendency was also seen in the ankle extensor.

#### Muscular activity during the stepcycle

Fig. 3 shows the EMG activity for a number of muscles in the chronic spinal cat and for comparison recordings from intact cats walking on the same treadmill under the same conditions. In the Result section we will however only deal with the results from the spinal preparations.

**Extensor muscles.** The ankle extensors had an intense onset during  $E_1$  with a maximal activation around the shift  $E_1-E_2$ . The intensity of the burst then continuously decreased to terminate in the end

of  $E_2$  (see Fig. 1). The general shape of the  $E_1$  curve was invariant with speed though the peak intensity increased. No distinct differences were found between the different ankle extensors (Sol) and the foot extensor (FDL) regarding timing or the shape of the bursts. In the recordings from some kittens still walking well a burst occurred in LG only at the transition of  $E_1-E_2$  corresponding to the usual peak of the EMG burst.

The pattern was different in the knee extensor. At lower speeds Q started smoothly during  $E_1$  and continuously increased until the end of  $E_1$  when it abruptly terminated (Fig. 3 and 4A and C). At the speeds the pattern changed entirely. A small burst appeared in the beginning of  $E_1$  or the end of  $E_1$ . The burst was usually isolated from the more rapid wave formed and much larger burst during  $E_2$  (Fig. 4B and C). The separation of the Q-burst in two bursts could be found in all kittens and in the only pattern seen in some even at slow walk. In a few kittens the second burst was very short and occurred at the end of

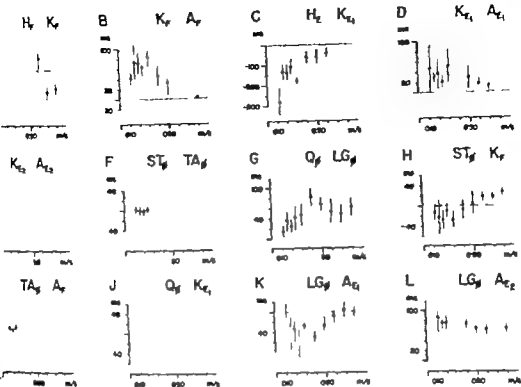


Fig. 3. Correlation of different movements and electromyographical data at different speeds. The onset of the flexion and extension phases in different joints and the onset of the locomotor bursts in different muscles are correlated to each other. The mean and S.D. of the differences are plotted in ms at different speeds. All data are from one spinal kitten. The experimental session (the same as in Fig. 2) and the observations for each data point vary between 8 and 14 trials. The experimental sessions used are H<sub>2</sub> (hip), K<sub>2</sub> (knee), A<sub>2</sub> (ankle) and the muscles F<sub>2</sub>, E<sub>2</sub> and E<sub>2</sub> the onset of the flexion, the first and the second extension phases respectively. ST<sub>2</sub>, TA<sub>2</sub>, Q<sub>2</sub> and LG<sub>2</sub> are abbreviations.  $\odot$  indicates the onset of the locomotor bursts. The muscles A thus show less the hip flexion occurs in relation to the knee flexion. A positive value means that the flexion precedes knee flexion. A-E show the onset of different phases of movements at different joints in relation to each other and F and G the onset of the flexion and extension of the knee and ankle in relation to each other. At higher speeds the onset of Q is counted from the first Q burst. H-L show the relation between muscle activity and movement.

the hip extensors studied gluteus medius and gluteus maximus had smooth onset during E<sub>2</sub>. The activity was usually maximal during E<sub>2</sub> but the onset of the activity was more variable than in the distal extensor muscles (see Fig. 3).

**Distal extensor muscles.** The flexors of the ankle (TA) and the knee (St) were usually synchronously activated during the very end of E<sub>2</sub> (Fig. 3 and 5 F). St had a very sharp onset and lasted only during the first part of F. TA had somewhat smoother onset and was active throughout F. A second burst of activity was found in St during E<sub>2</sub> at slower walks (about 40% of the cat) (see also Forsberg et al. 1970, Fig. 1 A). The size of the second burst increased with speed and at least a small peak of activity could be seen in all spinal preparations at

the fastest speeds (Fig. 4 B). A second burst or peak of activity during E<sub>2</sub> was never found in TA.

The hip flexor IP started more gradually than the distal flexor muscles to reach a maximum later in the stepcycle and cease approximately at the end of E<sub>2</sub>. Sartorius started later but reached its peak at the same time as IP. In some kittens an additional small burst more or less isolated from the main burst could occur at the end of E<sub>2</sub>.

The short foot dorsiflexor (EDB) displayed a large burst in E<sub>2</sub> and some residual activity throughout E<sub>2</sub> and E<sub>3</sub> (Fig. 3).

**Coordination of antagonistic muscles.** An apparent finding was the strict reciprocal activation of the extensor and the flexor muscles of the knee. The sharp termination of Q at the end of E<sub>2</sub> was

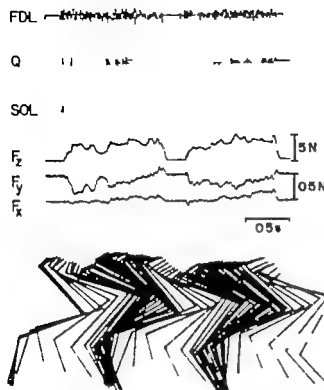


Fig. 6 Reaction forces produced during overground locomotion. The spinal kitten is held in a way that the right hindlimb is stepping on the forceplate. The movements are displayed as a stick diagram (see Fig. 1A) where the pelvis is displayed at a constant speed forward (to the right) synchronized to the recordings of the reaction forces and of the EMGs.  $F_z$  is the vertical force. The horizontal forces are divided into longitudinal components ( $F_y$ ) where the forwardly directed reaction force is displayed upwards and in transversal components ( $F_x$ ) where left is upwards. Note time and force calibrations.

always followed by as sharp an onset of St with a delay of 5–20 ms independently of the speed. St was never activated before the activity in Q had ceased (unfiltered EMGs). The shift during E from St to Q-activity was not as constant. At slow walks there was a longer delay (120 ms at 0.17 m/s) than in faster (15 ms at 0.80 m/s). The second smaller burst of St which occurred more regularly at higher speeds had a tendency to occur during the pause which separated the first Q-burst from its main burst.

There was not as distinct a reciprocity between the antagonists of the other joints. TA started after the smooth termination of LG. During E, however, LG could be activated before TA had ceased (Fig. 1C). Also in the hip joint there was an overlap of flexor and extensor activity.

### *Relations between the muscular activity and the movement at different joints and at different speeds*

During slow walk the onset of flexion of the hip and knee started approximately simultaneously. During slow walk the onset of flexion of the hip and knee started approximately simultaneously although there was a large variability (Fig. 1C). In the ankle joint the shift from extension to flexion was more distinct (Fig. 1C, 4A) but it occurred 40–80 ms after the flexion of the knee at slow walk (Fig. 5B). The flexor muscles of the knee and the ankle were however activated almost simultaneously (see above and Fig. 5F). The ankle flexion started 20–40 ms after the onset of the activity in the flexor muscle (Fig. 5I) i.e. the latency needed for effective electromechanical delay (Forsberg 1979). The knee however started to flex prior to activity in Q had ceased and that in St started (Fig. 4A and 5H). This means that the ankle joint followed the centrally generated efferent copy while the knee joint went into flexion despite ongoing extensor activity. The knee extensor torque thus not sufficient to counteract the external load (see Discussion). At faster walks the knee flexors could however contribute to the onset of flexion of the knee (Fig. 5H) as St-activity then started during the flexion. There was a more distinct extension during E<sub>2</sub> and a sharper transition into flexion (Fig. 4B). The delay between knee and ankle flexion practically abolished at the highest speed (Fig. 5L).

The knee and the ankle joint went into extension again in the middle of the swingphase and also in this shift the knee was leading (Fig. 5D). The knee extensor was as a rule not activated before the ankle extensor (Fig. 5K) and the knee extensor only at the highest speeds (i.e. the first Q burst, Fig. 5J), which indicates that the extension in both knee and ankle at least at lower speeds may be passively induced by the inertia of the moving limb. The hip kept flexed during the largest part of E and its extension occurred after the knee (Fig. 5C) and after the termination of activity in the extensor muscles (Fig. 1C).

When the foot was placed on the belt both knee and ankle was seemingly prevented from any further extension and as one would expect E<sub>2</sub> started simultaneously in the two joints (Fig. 5E). The ankle extensors were always active at least 40 ms prior to the placement of the foot at all speeds (Fig. 5L) i.e. well in time to meet the external load.

load of the body (see Discussion). When Q was suddenly activated at slow walks, the onset of  $E_1$  was always preceded by  $E_2$  (Fig. 4C). When stimulated with a first isolated small burst, in  $E_1$  or even in  $E_2$ , the larger burst was usually initiated around the placement of the foot, but in some cats regularly be activated 5–15 ms before foot contact (Fig. 4C).

**Terrestrial locomotion.** It may be asked if the limbs in addition to carry the weight of the cat can produce any propulsive force. An unspecific stimulation of the skin such as touching the tail somewhat. It was possible to evoke the locomotor activity (cf. Sherrington 1910). In Fig. 6 the front part of the body was carried by the tail, and slightly moved forward in a way that allowed the right hindlimb to be placed on a force during the support phase. The extensors produced force enough for one limb to carry most of the weight of the hindpart (55% of the total body weight). In spite of the large vertical force the body moved forward at the end of the swing phase and at the beginning of the support phase. This was due to an asymmetry of the two limbs where the contralateral was weaker than the recorded one and could not produce sufficient force. During the beginning of the support phase the limb was decelerated as a result of the backwardly directed reaction force. The forwardly directed reaction force, which in normal locomotion propels the animal forward, was, however, very small and could only be induced after stimulation of the tail. It is also noteworthy that the spinal catens could drive the cat on a belt at higher speed when the friction between the belt and the cat were balanced.

## DISCUSSION

In the intact animal increases its speed of propulsion. It might do so by progressively enhancing the intensity of activity in the neurons responsible for initiating the locomotor activity. Thus, in microstimulation preparations locomotion may be initiated from particular regions in the brainstem. Low frequency stimulation (e.g. at 50 Hz) may result in locomotion at slow walking speed. If the limbs are on a treadmill, the speed of locomotion may be varied within a considerable range by changing the belt speed. If the strength of the brainstem stimulation is increased the cat may trot and if further increased, gallop. The extra force needed is gener-

ated by recruitment of new motor units (Shik et al. 1966, Severin et al. 1967). The situation in the spinal cat is fundamentally different as the excitability of the cord cannot be modified by descending signals. In these animals the relevant spinal circuits have a constant excitability that clearly is high enough to elicit locomotion when the treadmill is driven within a rather large range of treadmill speeds. It is possible that the extra unspecific afferent input caused by the movements may give some extra drive to the generator network (see Grillner & Zangger 1979). It is reasonable to regard the spinal network as set for a very low speed, which is also in accordance with the finding that the hip extensor torque is small (Fig. 6). The fact that the movements adapt to the speed of the treadmill must rely on feedback from the moving limbs. There are several peripheral inputs that can serve to regulate the duration of different phases of the step (Pearson & Dussan 1976, Grillner & Rossignol 1978, Anderson et al. 1978).

The striking dependence on speed of the duration of the support phase (see Fig. 2) must thus be accomplished by the reflex input presumably in a similar way to that occurring in the intact animal (Arshavsky et al. 1965, Goslow et al. 1973). The yield of the knee and the ankle that occurs in the support phase is to a large extent due to the load on the limb that in the intact cat becomes maximal at the peak of the yield (Phillipson 1965, Grillner 1972, cf. Walmsley et al. 1978). The torque value around a joint at any instance depends i.a. on the load on the limb and the distance between the joint axis and the resultant of the force vectors (inertia neglected). The muscles should produce an equal torque opposing the external forces. It is clear that any effects such as a slight deviation of the resultant vector may lead to a marked change in the torque required. One minor abnormality of the spinal catens (at low speeds) is the fact that the knee flexion at the end of the support starts too early, i.e. before the knee flexors have started to become active. This may be due either to that the required torque is higher than normal (see above) or that the degree of extensor activity is too low.

It is interesting that the duration of the flexion phase decreases slightly with speed. To accomplish this the flexion torque must increase with speed particularly as progressively more torque will be required to reverse the direction of the entire limb. This results from a marked increase in the degree of flexor activation (Fig. 5). What induced this in-



crease in flexor activity? It would seem probable that it is related to events in the ipsilateral rather than the contralateral limb. If so it must be a process that occurs prior to the end of the St-burst and presumably before it has started. It is conceivable that events in the preceding phase could be the determining factor such as the duration of the support phase or the interval of the extensor activity or the velocity of extension.

There was also a substantial lengthening of the amplitude of the leg movement with speed in the spinal kittens. Such a lengthening may however also occur in intact cats. Calculations of the support length (i.e. the distance the animal moves during each support phase) from the graphs of Goslow et al. (1973) reveal a lengthening of 18% from slow walk (0.5 m/s) to fast walk (0.9 m/s) and even 35% to fast trot (1.7 m/s) but their later study (Wetzel et al. 1976) comparing only rather fast walks (1 m/s) and trots showed very small differences. Lengthening occurred also at low speeds in our intact cats walking on the same treadmill as the spinal kittens (40% from 0.1 m/s to 1 m/s) and in intact dogs (Arshavsky et al. 1965: 28% from 0.9 m/s to 3 m/s).

### Muscle activity

The intramuscular bipolar recordings performed have usually been rectified and filtered and related to the limb movements with the cats walking on treadmill at different constant velocities. This has allowed averaging of several consecutive cycles and thus a detailed comparison of onset and termination of the different locomotor bursts and the shape of the envelope of these bursts. We can thus compare the relative degree of activity in the step cycle between different preparations. It is however more difficult to compare the absolute level of activity in the different muscles as the recording conditions vary considerably with e.g. electrode placement in the muscle. To be able to compare spinal and intact cats, both types of preparations were tested on the same treadmill under the same recording conditions.

(a) *Knee flexors* (eg. St) are characterized by a short lasting burst in early flexion with a sudden onset and cessation (Engberg & Lundberg 1969; Grillner & Zangger 1975; Rasmussen et al. 1978; Wentink 1976 and Fig. 3B). In addition there is a second, more variable and smaller burst with its peak in the transition between F and E<sub>2</sub>. Both bursts grow with increasing speed. The spinal ani-

mal can reproduce this pattern, but in some cases the second burst appears only at higher velocities.

(b) *Ankle flexors* (eg. TA) have one burst per cycle that normally starts somewhat after the knee flexors and are of longer duration (Rasmussen et al. 1978 (intact); Gambian 1971; Grillner & Zangger 1975 (decerebrate)). The burst shows a sudden increase of activity which subsequently slowly declines (Fig. 3B). This pattern is at variance with Engberg & Lundberg (1969) who, based on monopolar recordings, concluded that knee and ankle flexors have a similar pattern. The exact pattern (Fig. 3) is mimicked by the spinal extensor at the onset of TA in our spinal cats usually a short time later than the knee flexors. This may however also occur also in intact preparations (not Rasmussen et al. 1978). Spinal curarized animals and locomotor bursts may however display differences in onset (Grillner & Zangger 1979; Arshavsky et al. 1978).

(c) *Toe flexors*. The long toe flexor (EDB) behaves as TA both in intact and spinal animals. The short toe flexor (EDB) on the contrary has a brief burst during E<sub>1</sub> and some residual activity throughout the support phase in both intact (Engberg & Lundberg 1969) and decerebrate (Grillner & Zangger 1975) and spinal preparations (Fig. 3).

(d) *Hip flexors* (IP Sart (medial part) and LG Sol) gradually at the same time approximate and gradually reach a maximum in the mid E<sub>1</sub> and then slowly decline in both spinal and intact cats (see Fig. 3) in contrast to TA which drops rapidly. The overall duration of the activity is thus longer (see Engberg & Lundberg 1969) and the envelope is different from the more distal flexors. These findings differ partly from the reports of similarity between TA and the hip muscles in Rasmussen et al. (1978) and in the mesencephalic cat Gambian et al. (1971; Grillner & Zangger 1975) and Perret (1973; Perret & Cabelguen 1979). In all cases, however, there is a clear difference between ankle, toe and hip flexors on one hand and knee flexors on the other.

(e) *Ankle and toe extensors* (LG Sol, EDL of mid E<sub>1</sub> to reach their maximum just before toe contact and then slowly decline and cease with time before end of F<sub>1</sub> (Fig. 3) in agreement with Engberg (1964), Engberg & Lundberg (1969), Rasmussen et al. (1978) and Walmsley et al. (1977).

(f) *The knee extensor* (vastus lateralis or

can display two different patterns in both directions. At low velocities a continuous burst smoothly at foot contact and increases progressively to be maximal at the end of  $E_3$  (Fig. 4C). At higher there is instead an early small burst at end of F and a subsequent sudden increase in the main burst starting just at foot contact (see however below). The transition from one type of pattern to the other is gradual. The nature of this shift from a functional point of view is not clear and neither what neuronal mechanisms are responsible (cf. however the short ST in  $E_1$ - $E_3$  that increases with speed). The second appears somewhat delayed in the spinal cat. The only pattern described previously (Zangger & Lundberg 1969) is in accordance with found in faster locomotion.

The hip extensor compared is gluteus medius.

There is no clear difference between the

and the intact pattern.

**Torque and muscle activity.** One consistent

is a difference in the envelope of the recorded

EMGs of the knee and ankle extensors (see

Fig. 3). The ankle extensors displayed an intense

and a successive reduction (cf. also Walmsley

1978), while the knee extensor had a smooth

(at low speeds) and a steady increase until a

cessation. The torque of the knee is low dur-

ing the first part of the support phase (Mänter 1938

refer. unpubl.) whereas in the ankle the torque

required muscle force increase rapidly to its

maximum (Mänter 1938, Grillner 1977, Walmsley et

al. 1978, Zomlefer unpubl.). The relative require-

ments of muscle force in different periods of the

support phase are thus similar to the shape of the

bursts of the knee and ankle extensors.

**Striking remarks.**

Though the spinal cat may have several disabling

defects in relation to the overall control of locomotion

(e.g. equilibrium deficiencies, adductor tones,

asymmetries, the striking finding is that the

spinal cord with its reflex machinery may still re-

produce even very subtle details of the basic walk-

movements of the intact cat. The duration of

the support phase is obviously controlled by

external input. It is however unlikely that the timing

of the different EMG-bursts depend causally on

afferent reflex input from the moving limb as deaf-

ened or paralyzed mesencephalic cat can still

produce this complicated pattern (Grillner & Zang-

ger 1975, Perret & Cabelguen 1979). It should be recalled that the output pattern of a cat with isolated spinal cord (i.e. with dorsal roots and spinal cord transected) has so far not been shown to produce as complex a pattern, although it is more complicated than a flexor-extensor alternation (Grillner & Zangger 1979). In addition mesencephalic cats have a supplementary phasic neuronal input from the vestibulo-rubro- and reticulospinal tracts (Orlovsky 1970, 1972a, b), that may even influence the spinal pattern generating network (Russell & Zajac 1979).

The overall picture is nevertheless that the spinal cord may contain a very significant part of the pattern generating circuitry for locomotion. The findings are thus compatible with the view that a simple descending control signal (such as an unpatterned spike train) could normally initiate locomotion by inducing activity in networks located within the spinal cord. Such spinal circuits may indeed operate also in the normal adult cat as locomotion can be induced by noradrenergic precursors or receptor stimulators in acute spinal cats (Jankowska et al. 1967a, b, Grillner 1969, Forsberg & Grillner 1973, Budakova 1973).

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mal can reproduce this pattern but in some the second burst appears only at higher velocities.

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(e) *Ankle and toe extensors* (*LG Sol*, *FDL*) in mid  $E_1$  to reach their maximum just before contact and then slowly decline and cease some time before end of  $E_1$  (Fig. 3) in agreement with Engberg (1964), Engberg & Lundberg (1969), Rasmussen et al. (1978) and Walmisley et al. (1978).

(f) *The knee extensor* (*vastus lateralis*) or

cat can display two different patterns in both phases. At lower velocities a continuous burst smoothly at foot contact and increases progressively to be maximal at the end of E. (Fig. 4C). At higher there is instead an early small burst at end of F and a subsequent sudden increase thirty in the main burst starting just at foot contact (see however below). The transition from one type of pattern to the other is gradual. The chance of this shift from a functional point of view is not clear and neither what neuronal mechanisms are responsible (cf. however the short ST in  $E_1-E_2$  that increases with speed). The second burst appears somewhat delayed in the spinal cat. The only pattern described previously (berg & Lundberg 1969) is in accordance with burst in faster locomotion.

The hip extensor compared is gluteus medius (Fig. 3). There is no clear difference between the two and the intact pattern.

**Torque and muscular activity.** One consistent finding was a difference in the envelope of the recorded EMGs of the knee and ankle extensors (see Fig. 3). The ankle extensors displayed an intense and a successive reduction (cf. also Walmsley 1977), while the knee extensor had a smooth increase (at lower speed) and a steady increase until a cessation. The torque of the knee is low during the first part of the support phase (Münster 1938, Zomlefer unpubl.) whereas in the ankle the torque required muscle force increase rapidly to its maximum (Münster 1938, Grillner 1972, Walmsley et al. 1977, Zomlefer unpubl.). The relative requirements of muscle force in different periods of the support phase are thus similar to the shape of the bursts of the knee and ankle extensors.

**Concluding remarks.** Though the spinal cat may have several disabilities in relation to the overall control of locomotion, e.g. equilibrium deficiencies, adductor tonus, asymmetries, the striking finding is that the spinal cord with its reflex machinery may still regulate even very subtle details of the basic walking movements of the intact cat. The duration of the support phase is obviously controlled by sensory input. It is, however, unlikely that the timing of the different EMG-bursts depend causally on proprioceptive reflex input from the moving limb as deafened or paralyzed mesencephalic cats can still produce this complicated pattern (Grillner & Zangger 1974, Perret & Cabelguen 1979). It should be recalled that the output pattern of a cat with isolated spinal cord (i.e. with dorsal root and spinal cord transected) has so far not been shown to produce a complex pattern although it is more complicated than a flexor-extensor alternation (Grillner & Zangger 1979). In addition mesencephalic cat has a supplementary phasic neuronal input from the vestibulo-, rubro- and reticulospinal tracts (Orlovsky 1970, 1977a, b) that may even influence the spinal pattern generating network (Russett & Zajac 1979).

The overall picture is nevertheless that the spinal cord may contain a very significant part of the pattern generating circuitry for locomotion. The findings are thus compatible with the view that a simple descending control signal (such as an unpatterned pulse train) could normally initiate locomotion by inducing activity in network located within the spinal cord. Such spinal circuit may indeed operate also in the normal adult cat as locomotion can be induced by noradrenergic precursors or receptor stimulants in acute spinal cat (Jankowska et al. 1967a, b, Grillner 1969, Forssberg & Grillner 1973, Budakova 1973).

The excellent help of Mrs Margret Svaneberg, Maria Widen and Lager Klingebjörn is gratefully acknowledged. Dr Anders Spjutrom took part in the animal experiments. The work was supported by the Swedish Medical Research Council, project no. 14X 0036 and from Karolinska Institute J 11, as supported in part by the Delft University of Technology, the Netherlands.

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crease in flexor activity? It would seem probable that it is related to events in the ipsilateral rather than the contralateral limb. If so it must be a process that occurs prior to the end of the St burst and presumably before it has started. It is conceivable that events in the preceding phase could be the determining factor such as the duration of the support phase or the interval of the extensor activity or the velocity of extension.

There was also a substantial lengthening of the amplitude of the leg movement with speed in the spinal kittens. Such a lengthening may however also occur in intact cats. Calculations of the support length (i.e. the distance the animal moves during each support phase) from the graphs of Goslow et al. (1973) reveal a lengthening of 18% from slow walk (0.5 m/s) to fast walk (0.9 m/s) and even 35% to fast trot (2.7 m/s) but their later study (Wetzel et al. 1976) comparing only rather fast walks (1 m/s) and trots showed very small differences. Lengthening occurred also at low speeds in our intact cats walking on the same treadmill as the spinal kittens (40% from 0.1 m/s to 1 m/s) and in intact dogs (Arshavsky et al. 1965: 28% from 0.9 m/s to 3 m/s).

#### Muscle activity

The intramuscular bipolar recordings performed have usually been rectified and filtered and related to the limb movements with the cats walking on treadmill at different constant velocities. This has allowed averaging of several consecutive cycles and thus a detailed comparison of onset and termination of the different locomotor bursts and the shape of the envelope of these bursts. We can thus compare the relative degree of activity in the step cycle between different preparations. It is however more difficult to compare the absolute level of activity in the different muscles as the recording conditions vary considerably with e.g. electrode placement in the muscle. To be able to compare spinal and intact cats both types of preparations were tested on the same treadmill under the same recording conditions.

(a) *Knee flexors* (eg. St) are characterized by a short lasting burst in early flexion with a sudden onset and cessation (Engberg & Lundberg 1969; Grillner & Zangger 1975; Rasmussen et al. 1978; Wentink 1976; and Fig. 3B). In addition there is a second more variable and smaller burst with its peak at the transition between E and E<sub>2</sub>. Both bursts grow with increasing speed. The spinal ani-

mal can reproduce this pattern but in some the second burst appears only at higher velocities.

(b) *Ankle flexors* (eg. TA) have one large cycle that normally starts somewhat after the knee flexors and are of longer duration (Rasmussen et al. 1978; (intact); Gamberian 1971; Grillner & Zangger 1975; (decerebrate)). The burst shows a small increase of activity which subsequently declines (Fig. 3B). This pattern is at variance with Engberg & Lundberg (1969) who from monopolar recordings concluded that late ankle flexors have a similar pattern. The intact pattern (Fig. 3) is mimicked by the spinal except that the onset of TA in our spinal cats usually is not simultaneous with the knee flexors. This may however occur also in intact preparations (for Rasmussen et al. 1978). Spinal curarized animals and locomotor bursts may however display differences in onset (Grillner & Zangger 1979; Arshavsky et al. 1978).

(c) *Toe flexors*. The long toe flexor (EDL) has as TA both in intact and spinal animals. The short toe flexor (EDB) on the contrary has a big burst during E and some residual activity throughout the support phase in both intact (Engberg 1969; decerebrate) (Grillner & Zangger 1975) and spinal preparation (Fig. 3).

(d) *Hip flexors* (IP Sart (medial part)) rise gradually at the same time approximately as the ankle flexors (b) to reach a maximum in the end of E and then slowly decline in both spinal and intact cats (see Fig. 3). In contrast to TA it drops rapidly. The overall duration of the activity is thus longer (see Engberg & Lundberg 1969) and its envelope is different from the more distal flexors. These findings differ partly from the reports of timing between TA and the hip muscles in Rasmussen et al. (1978) and in the mesencephalic cat Gamberian et al. 1971; Grillner & Zangger 1975; Perret 1973; Perret & Cabelguen 1979). In all cases however there is a clear difference between the toe and hip flexors on one hand and knee flexors on the other.

(e) *Ankle and toe extensors* (LG Sol, EDL<sub>2</sub>) in mid E to reach their maximum just before contact and then slowly decline and cease a little time before end of E (Fig. 3) in agreement with Engberg & Lundberg (1964); Engberg & Lundberg (1969); Rasmussen et al. (1978) and Walmisley et al. (1973).

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can display two different patterns in both phases. At lower velocities a continuous burst smoothly in foot contact and increases slowly to be maximal at the end of E (Fig. 4C). At higher there is instead an early small burst at end of F and a subsequent sudden increase half in the main burst starting just in foot contact (see however below). The transition from one type of pattern to the other is gradual. The cause of this shift from a functional point of view is not clear and neither what neuronal mechanisms are responsible (cf. however the short ST in E<sub>1</sub>-E<sub>2</sub> that increases with speed). The second appears somewhat delayed in the spinal cat. The only pattern described previously (e.g. Lundberg 1969) is in accordance with the need for faster locomotion.

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# Locomotion of the low spinal cat Interlimb coordination

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The interaction of the two hindlimbs were investigated by an analysis of the muscular activity and the movement in 14 chronic spinal kittens during treadmill locomotion (in kittens subjected to transection of the spinal cord (Th<sub>12</sub>) one or two weeks after birth). At low speed the limbs were alternating (walk or trot). At higher they were activated more simultaneously as during gallop. The two limbs could walk at different velocities as during walking in circle when the two belts of the treadmill were driven at different speed. The duration of the support phases was mainly influenced by the speed of the belt on which the limb was walking. The limbs could still maintain common rhythm up to a two or three fold speed difference in the flexion or the first extension phase of the limb walking on the "fast" belt, in prolonged and the flexion phase of the slow limb was shortened. At extreme speed differences the limb on the "fast belt" performed 3 and even 4 steps during one step cycle of the slow limb. The placement of the feet was found to maintain the most stable relationship during alternating gait at different speed differences. It is concluded that all phases of the step cycle are modifiable and that there are several mechanisms coordinating the limbs rather than the spinal cord.

**Key words:** Locomotion, spinal cord, spinal transection, interlimb coordination.

Holst (1934, 1935, 1939) suggested on the basis of series of elegant studies that the coordination between the different fins during fish locomotion arose from an interaction between independent neural centers controlling each fin. Von Holst (1939) also pointed to the possibility that this principle could apply to the coordination of the limbs in mammals. This idea was developed further in a critical model of quadrupedal locomotion (Oeltdorf et al. 1966), which in turn was supported by experimental evidence (Sluk & Orlovsky 1963, Hagen & Slak 1970, Grillner & Zangger 1979) structures, which may be responsible for the coordination between different generators have been identified in two invertebrate motor systems, the controlling walking in the cockroach and the lateral swimmeret system of the crayfish (Pearson & Des 1973, Stein 1971, 1974, 1976). These neurons are active during the segmental motor bursts and provide an efference copy which is somehow used to achieve intersegmental coordination in the swimmeret system studies on the effect of indirect activation of the coordinating

neurons have allowed the identification of certain properties of the coordinating system (Stein 1974, 1976). Also in mammals (cat) part of the ascending neuronal activity to the cerebellum provides efference copy signals (Arshavsky et al. 1972, 1978). Such signals could also be utilized to coordinate different autonomous networks of the limbs (Grillner 1975). Until now there is however no mammalian work that so far has revealed the specific neuronal mechanisms of the interlimb coupling. Even on behavioural level little is known on the mode of interaction between these networks in different situations and in different animal preparations.

The spinal cord of the cat has the capacity to produce the detailed coordination of different muscle groups of a limb which results in walking.

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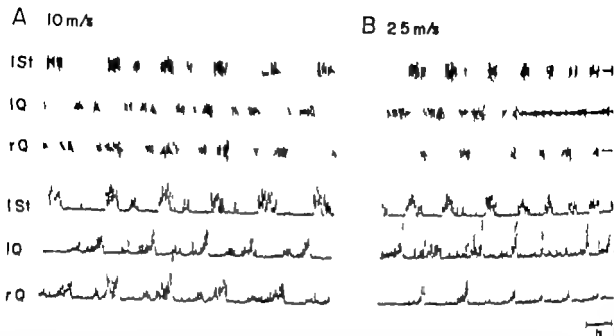


Fig. 1. Muscular activity of a chronic spinal cat during treadmill locomotion. The raw (amplified and high-pass filtered) EMGs from the flexor (IS) and the extensor (IQ) of the left knee and from the right knee extensor (RQ) are shown at the top and the rectified and filtered versions are shown at the bottom. A: Alternating locomotion at a speed of 1 m/s. Change over from an alternating to a nonalternating pattern at a speed of 2.5 m/s. (From Tiger)

movements (Forsberg et al 1980). To induce such movements the supraspinal centers may only have to regulate the excitability level of the relevant spinal network. But in addition each limb must be coordinated to all the other limbs. In normal locomotion along a straight line, despite large variations, two main forms of hindlimb coordination can be observed: alternate mode as in walking or trotting or a more or less simultaneous mode as in the different forms of gallop (Stuart et al 1973; Halberstam et al 1976; English 1979). Another complex form of interlimb coordination occurs when an animal walks along a circular path and the outer limbs have to cover a longer distance (i.e. walk "faster") than the inner ones. The mesencephalic walking cats deal with such a situation by appropriate adaptations of the stepcycles on the two sides (Kulagin & Shik 1970). This was studied by having the cats walk with the right and the left pair of limbs on two treadmill belts, the speed of which could be controlled independently.

The present work describes how the lumbosacral spinal cord alone, in chronic spinal cats, manages to coordinate both hindlimbs when walking at different speeds under the situations discussed above. Preliminary reports have been published (Forsberg, Grillner and Sjöström in Grillner 1973 and Forsberg et al 1976).

## METHODS

Four low ( $\text{Th}_6\text{--Th}_{11}$ ) chronic spinal cats were used as the main part of this study, but additional observations were also made on 10 more similar spinal cats. The species, the training procedures and the recordings and data processing techniques were described fully in Forsberg et al (1980). In four of the cats the movements and electromyograms (EMGs) of the two hindlimbs were recorded during locomotion on a treadmill with "split belts". Both belts could be driven at the same speed or one could be disconnected from the motor and manually operated; the other was still driven by the motor. The speed of the other was made to walk at different speeds. The velocity of the belts was not always constant, especially at lower speed, and was therefore also estimated by movements of the diodes on the transverse distance during the support phases. The limb walking on the fast belt is called the "fast limb" and that on the slow belt the "slow limb".

**Abbreviations.** The following abbreviations are used: Flexion phase: F; first, second and third extension phase:  $\text{F}_1$ ,  $\text{F}_2$  and  $\text{F}_3$  respectively; m: tibialis anterior;  $\text{Ta}$ : quadriceps (vastus lateralis); Q: m. semitendinosus;  $\text{Sd}$ : ST m. iliopsoas; IP: m. sartorius; S: standard deviation; ED: electromyogram; EMG.

## RESULTS

### 1. Treadmill locomotion

Chronic spinal cats could walk on a treadmill at a speed adjusted to that of the treadmill belt and with

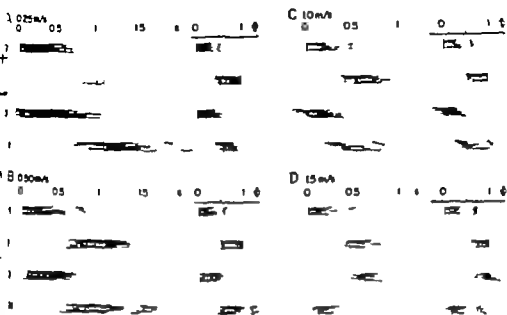


Fig. 6. Schematic representation of the muscular activity from a chronic spinal cat during treadmill locomotion at various speeds. The duration of the EMG-bursts of left 1st, and 1Q and right 1st and 1Q are measured from graph recordings on a graphic tablet (digitizer) connected to a desk calculator (HP 9910 A) and by a plotter drawn in real time to the left (note time scale at the top) and normalized to the cycle duration to the right. The bursts of the muscles from several consecutive stepcycles are plotted beneath each other. A cycle starts with the onset of 1st. The end is marked with a single dot. A, B and C show alternating gaits at increased speeds. D shows locomotion pattern as during gallop. (From Tiger.)

extensor pattern of each limb similar to that of intact cats (Forsberg et al 1980). During slow walk the two limbs alternated, i.e. an event in one limb was delayed out of phase by half a stepcycle with the same event in the other limb (Figs 1A and 1B and C). In Fig. 6 (left column) the onset of the midpoint of the support phase (A) and of the extension (B) and flexion (C) of the hip of the hindlimbs are out of phase by 50%. The onset of the termination of the extensor activity were, however, not as symmetric (Fig. 6D: left column). This asymmetry was due to a difference in the burst duration (20% shorter in the left limb). However, the midpoints of the period of support of the two limbs were alternating (close to 50% cf. also 6A and 1B). The asymmetries between the limbs were common in all spinal cats but at different degrees and they could be more or less pronounced than is shown in Fig. 6. Behaviourally such asymmetries were manifested as limping (cf. Forsberg et al 1980). All cats ( $n=14$ ) displayed alternation over a wide range of velocities (cf. Fig. 2A-C). When the speed increased they ( $n=11$ ) could sud-

dently switch to another stable mode of interlimb coordination in which the two limbs performed simultaneous movements (Fig. D). The activity in the two limbs was not synchronous but one limb was usually leading the other with a rather stable lag (Fig. 1B cf. also Fig. 3 in Grillner 1973 and Cohen 1979). Some cats could maintain long periods of stable hindlimb gallop while others performed only 5-6 steps and then reverted to alternation. The readiness to respond with gallop changed with time so that they could exhibit stable gallop at a very low speed at one time and some what later only at a much higher treadmill velocity. Long term changes were also observed.

## II. Locomotion on split belts

1. *Effects on different part of the stepcycle* The duration of the stepcycles decreases at faster walking speeds (Forsberg et al 1980 Fig. 2). From this it could be inferred that when two limbs in the same girdle walk at different speeds they should walk at different frequencies if the cycle duration was only determined by the walking speed. In Fig. 3

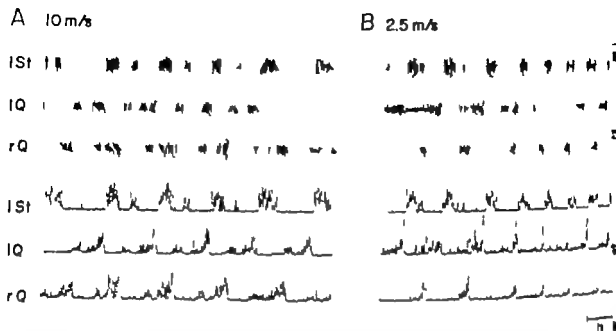


Fig. 1. Muscular activity of a chronic spinal cat during treadmill locomotion. The raw (amplified and high pass filtered) EMGs from the flexor (ISI) and the extensor (IQ) of the left knee and from the right knee extensor (rQ) are shown at the top and the rectified and filtered versions are shown at the bottom. A: Alternating locomotion at a speed of 10 m/s. Change over from an alternating to a nonalternating pattern at a speed of 2.5 m/s. (From Tiger.)

movements (Forsberg et al 1980). To induce such movements the supraspinal centers may only have to regulate the excitability level of the relevant spinal network. But in addition each limb must be coordinated to all the other limbs. In normal locomotion along a straight line, despite large variations, two main forms of hindlimb coordination can be observed: alternate mode as in walking or trotting or a more or less simultaneous mode as in the different forms of gallop (Stuart et al 1973; Halberstam et al 1976; English 1979). Another complex form of interlimb coordination occurs when an animal walks along a circular path and the outer limbs have to cover a longer distance (i.e. walk faster) than the inner ones. The mesencephalic walking cats deal with such a situation by appropriate adaptations of the stepcycles on the two sides (Kulagin & Shik 1970). This was studied by having the cats walk with the right and the left pair of limbs on two treadmill belts; the speed of which could be controlled independently.

The present work describes how the lumbosacral spinal cord alone in chronic spinal cats manages to coordinate both hindlimbs when walking at different speeds under the situations discussed above. Preliminary reports have been published (Forsberg, Grillner and Sjöström in Grillner 1973 and Forsberg et al 1976).

## METHODS

Four low ( $T_6$ – $T_{11}$ ) chronic spinal cats were used as the main part of this study, but additional observations were also made on 10 more similar spinal cats. The general training procedures and the recording and processing techniques were described fully in Forsberg et al (1980). In four of the cats the movements and electromyograms (EMGs) of the two hindlimbs were recorded during locomotion on a treadmill with split belts. The belts could be driven at the same speed or one could be disconnected from the motor and manually operated, the other was still driven by the motor. The two belts could thus be made to walk at different speeds. The velocity of the belts was not always constant, especially at lower speeds and was therefore also estimated for movements of the diodes on the transmetatarsal joint during the support phases. The limb walking on the fast belt is called the *fast limb* and that on the slow belt the *slow limb*.

**Abbreviations.** The following abbreviations are used: Flexion phase: F; first, second and third extension phase:  $E_1$ ,  $E_2$  and  $E_3$  respectively; m: tibialis anterior; TA: quadriceps (vastus lateralis); Q: m. semitendinosus; ST: m. iliopectineus; IP: m. sartorius; S: standard deviation; S II: electromyogram (EMG).

## RESULTS

### 1. Treadmill locomotion

Chronic spinal cats could walk on a treadmill at a speed adjusted to that of the treadmill belt and not

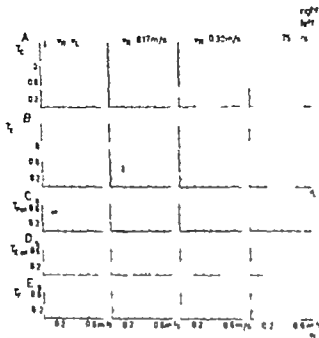


Fig. 4. Adjustments of the step cycle during locomotion on a split belt. One belt is driven at constant speed (the right motor driven belt) and the other driven at various speeds. The speed of the right belt is given at the top of each column; the speed of the left is along the abscissa in each plot. In the columns to the left the belts were "bed" together and driven at the same speed. (A, B, C and D) the durations of the step cycle ( $T$ ), the support phase ( $T_s$ ), the flexion phase ( $T_f$ ) and the first extension phase ( $T_e$ ) of both limbs are plotted versus the speed of the left limb. The durations are calculated from the angular movement in the knee or ankle (A and B) or from the ankle (C and D). (E) the duration of the flexion phase of the hips ( $T_h$ ) are plotted. The mean of the durations of the right limb is indicated by open circles and those of the left by filled circles (5-16). S.D. is indicated when it exceeds the circle. Some data points are missing due to difficulties in determining some phases from the angular movement (especially at the ankle). (From Mass.)

se were much shorter. To maintain the same duration either the step cycle might be proportionally changed, i.e. each phase would occupy the same portion of the step cycle (see Kulagin & Shikha, or one phase might be more influenced than others. From Table 1 it is seen that the proportion taken up by the support phase was changed when the speed of each belt was the same in the different conditions. The duration of the support phase had instead a tendency to preferentially influenced by the belt speed. When the speed of the fast limb (left) was increased (see Fig. 4B,  $V = 0.30$  m/s) the duration of the support phase was increased and followed a similar curve as during normal locomotion. If the speed of the "fast limb" (left) was held constant (see Fig. 4B,  $V = 0.75$  m/s) the duration of the support phase was only

slightly shorter when the speed of the "slow limb" was decreased. In the latter the duration of the support phase increased when the speed decreased (Fig. 4B,  $V = 0.75$  m/s) or decreased only slightly at constant speed when the "faster limb" walked faster (Fig. 4B,  $V = 0.17$  m/s and  $0.30$  m/s). There could however be substantial deviations (see Table 1 and below).

As a consequence of the diverging influences from the two limbs during "split belt" locomotion the duration of the support phase of the slow limb was always longer than that of the fast limb (see Table 1 and Fig. 4B).

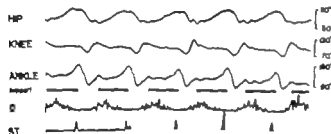
To maintain the same rhythm the differences in support phase had to be compensated for. Three different types of changes were recognized. (i) In the "fast limb" the swing phase was prolonged and

Table 1 Modification of the step cycle during split belt locomotion in four spinal cats

To the right the two limbs simultaneously walk at different speeds ("split belt"). To the left the limbs walk at the same speed ("tied belts"). The latter speeds are chosen to be the same as those of each split belt. The duration of the step cycle (in 1-4) and the real and relative ((support phase/step cycle)  $\times 100\%$ ) duration of the support phase in 1-4 is compared with regard to the same limb in each condition. Means and standard deviations are calculated in 1-4 and the data are compared by Student's *t*-test  $p < 0.05$  and  $p < 0.01$ . Note however that the variance of the belt speed is not measured in the different conditions. In Elsa (1) with a dominance of the left limb the data on the split belt is taken from two different sessions: first with the left limb walking on the slow belt and then with the left limb on the fast belt.

Tied belts					Split belt		
	Speed (m/s)	Cycle dur (ms)	$E_1 + E_2$ (ms)	$\tau$	Cycle dur	$E_1 + E_2$	$\tau$
1 Tiger	0.60	$151 \pm 60$			$1309 \pm 61$		
	0.90	$988 \pm 52$			$1314 \pm 67$		
Elsa	0.75	$1149 \pm 11$			$1109 \pm 50$	left	see legend
	1.00	$732 \pm 43$			$876 \pm 44$	left	
3 Liza	0.15	$1131 \pm 69$	$849 \pm 75$	75	$996 \pm 46$	$811 \pm 46$	85%
	0.50	$546 \pm 3$	$246 \pm 4$	46	$989 \pm 65$	$340 \pm 57^*$	34%
	0.15	$1111 \pm 69$	$849 \pm 75$	75	$753 \pm 105$	$490 \pm 71$	65%
	0.40	$620 \pm 45$	$313 \pm 53$	50	$75 \pm 81$	$764 \pm 37^{**}$	10%
4 Mina	0.17	$849 \pm 86$	$423 \pm 100$	50	$684 \pm 61$	$480 \pm 99$	70%
	0.74	$680 \pm 4$	$279 \pm 79$	41	$681 \pm 45$	$13 \pm 19^*$	2%

## SLOW LIMB (right) 0.30 m/s



## FAST LIMB (left) 0.70 m/s

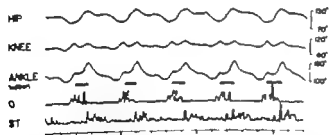


Fig. 3 Locomotion of a chronic spinal cat on split belt in a 1:1 rhythm. The angular movement of the hip, knee and ankle and the synchronously recorded rectified and filtered EMGs of Q and St are shown for both limbs. The support phases are indicated with bars. The belt on the right side was driven at 0.30 m/s and that of the left at 0.70 m/s. Angular calibrations are to the right of each curve and the time calibration is on the bottom in second (large bars) with 100 ms subdivisions (small bars). (From Mina)

the left limb was walking on a belt which was more than twice as fast as that of the right limb. It is still the two limbs walked at the same rhythm. Hence there are some spinal mechanisms which coordinate the cycles to equalize their duration. Table 1 the duration of the step cycles at different velocities during split belt walking" is compared with that during locomotion at the same speed on tied belts. Usually the cycle of the fast limb was prolonged and that of the slow shortened as seen in mesencephalic cats (Kulagim & Shik 1970). The degree of shortening and prolongation varied, however, between the different cats and also between different trials in the same cat. One of the limbs was 'dominated' usually and had a stronger influence on the rhythm. In one cat (Tiger) the slow limb dominated slightly (irrespective of left or right). In another (Mina) the fast limb had the most powerful influence and determined the rhythm. In Tiger the left limb was dominant whether it was the slow or the fast limb and finally in Liza either the slow or the fast limb could have the largest influence.

In Fig. 3 there are striking differences between the muscular activity and the angular movement of the two limbs. The extensor burst and the upper phase of the slow limb were much longer than those of the fast limb and the burst and flexor

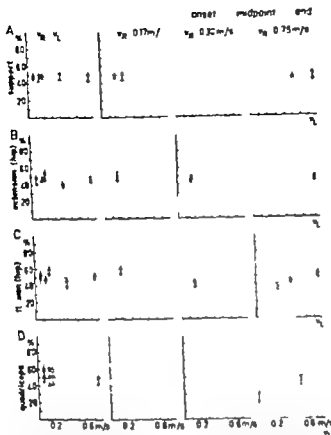


Fig. 6. Coordination between some events of the locomotor activity of the two limbs. The same experimental paradigm as in Fig. 4 is used. The speed of the right belt is above each column, the speed of the left is along the abscissa. The relative interval (interval/cycle duration)  $\times 100\%$  from the onset (triangles) or midpoint (squares) of the left limb to the corresponding event of the right limb is plotted for A: the support phase, B: the extension and C: the flexion phase of the hip and for  $\blacksquare$  the quadriceps activity. In D the relative interval between the termination of activity in the  $ra$  limbs is also plotted. The mean and S.D. are indicated ( $n = 5-16$ ). The relation of the movement of the knees and of the ankles changes as a function of  $v$ . (From Morin.)

Fig. 6). Even if the onset of the different limb change (might have been expected that midpoints of e.g. the support phases should remain in strict alternation (circles in Fig. 6) it should mean that whole phases would be reciprocally coupled. However this was not the case (Fig. 6).

The onset of the extensor activity (Fig. 6D) extended (Fig. 6B) and the placement of the feet (Fig. 6C) were nevertheless all relatively close to 50% of the cycle, e.g. the midpoints or terminations (Fig. 6D). The relations between the onset of flexor muscles or the onset of flexion (Fig. 6C) and substantially the different speed combina-

tions but in a linear way depending on the more constant extensor part and on the changed relative durations of the support phases (see above).

**3. Locomotion with different rhythms of the two limbs.** At extreme differences between the speed of the two belts the compensatory changes of the step-cycles (see sect. II.1) were not sufficient to maintain a common rhythm of the two limbs. Instead of a prolonged waiting in the swing phase, the 'fast' limb executed an extra step during the support phase of the slow limb (Fig. 7A). The occurrence of extra steps depended on the relative speed difference between the two belts but also on the leg asymmetries (see sect. I). At certain speed differ-



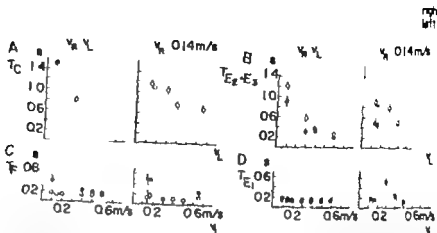


Fig. 5 Adjustments during locomotion on split belts. The same experimental paradigm as in Fig. 4 is used. The left part of each graph shows the situation when both belts are driven at the same speed. In the right part the right belt is driven at a constant speed of 0.14 m/s and the left belt driven at various speeds along the abscissa. The duration of A the stepcycle ( $T$ ) B the support phase ( $T_{12} - E_3$ ) C the flexion phase ( $T_1$ ) D the first extension phase ( $T_{11}$ ) as determined from the angular movement of the ankles are plotted against the velocity of the left limb. The mean and S.D. are plotted for both limbs ( $n=5-14$ ). Open circles are from the right limb and filled circles from the left. (From Liza.)

the limb "waited" in the last part of flexion or in the first extension phase (Fig. 3) (ii) In the "slow limb" the swing phase was shortened usually due mainly to a reduction of the flexion phase (Fig. 4C) with only small changes in the first extension phase (Fig. 4D) (iii) In the "slow limb" there could also be a substantial reduction of the support phase (Table 1.3). These three compensatory changes could be identified in all cats, but their relative importance differed depending on whether the "fast" or the "slow limb" dominated the rhythm (see above).

The influence from the "fast limb" to reduce the support phase of the "slow limb" (iii) would be opposite to that of the peripheral feedback from the "slow limb" which would act to maintain the duration of the support phase. In Fig. 5 one may recognize a shift in the balance of these conflicting influences. At the highest and the third highest speed of the left ("fast") limb the influence from the "fast limb" dominated with a shortening of the support phase of the "slow limb" (Fig. 5B) and of the common stepcycle duration (Fig. 5A). At the other speeds of the "fast limb" the cycle duration and the support phase of the "slow limb" remained constant (Fig. 5A, B). In the latter cases the "fast limb" had to adapt by waiting in flexion (Fig. 5C, 0.50 m/s) or first extension (Fig. 5D, 0.30 m/s) with a long duration of the stepcycle (Fig. 5A).

Coordination between the limbs: During strict

alternating locomotion (walk or trot) with symmetric stepcycles the muscular activity and the movements in one limb will follow the same events of the other limb after exactly half a step cycle. The events are thus shifted by "50°" although there may be some variability around the mean (Stern et al. 1973; English 1979; cf. Halbertsma et al. 1974). In the present split belt situation the relative duration of the different parts of the step cycle are unequal and thus there is no possibility for all events to remain 50% out of phase. If some events should be more tightly linked they should maintain a fixed phase relationship independently of other changes. Another mechanism would be a constant lag between two opposite events, e.g. flexion in one limb and extension in the other. No such constant lag was found, and in fact it could not be a possible mechanism since the relative duration of the different phases changes as the speed of locomotion varies (Goslow et al. 1973; Forsberg et al. 1980).

Fig. 6, left column shows how the onset and the midpoint of different phases remain in approximately 50% when the two limbs walk at the same speed (see also legend Fig. 6). In the 14 different combinations of speeds between the left and the right side shown in the three vertical columns to the right there is no indication that the onset of either support, extension, flexion or quadriceps EMG activity remain at one fixed phase relationship (the

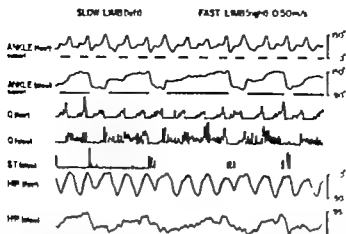


Fig. 8. Relative coordination ( $\sim 1$ ) of chronic spinal cat walking on split belt. The angular movement of the saddle and hip and the rectified and filtered EMGs are presented as in Fig. 3. The velocity of the slow belt used between 0.04 and 0.11 m/s. That of the fast belt is 0.40 m/s. (Note time and angular calibrations) (1 rom. 1.22 s).

or during 1 or 3 gait the extensors of soles co-contract during part of the step. A reciprocal apparent depression of the activity was often seen as in the 2-1 steps of 1A, then the extensor burst of the fast limb after when it occurs during the extensor burst of slow limb (cf. also Fig. 8). The a-coupled of the extensor activity of the slow limb (Fig. 7B) do also show depression in the beginning of the extensor burst. In the slow walk on split belt in Fig. 9A it is striking that the amplitude was much larger when no co-activation occurred. This reciprocal interaction was sometimes very apparent, especially in the knee extensors, and at other times not obvious. The exact basis is unknown. It may be a central one between parts of the generator network or its on the motoneurons or premotor interneurons. It may also be of reflex origin, although it should be noted that the depression usually started as the foot was placed on ground. Some signs of reciprocal effects between co-contracting extensors and flexors were also seen. The 2nd ST burst in Fig. 9B coincides with a phasic depression of the Q-burst. Such reciprocal relations have also been seen after deafferentation in walking vertebrate cats (Grillner & Zangger unpubl.) and did advocate a central origin of the effects. In some instances, during the 1 gait there are small flexor bursts in the slow limb during the

long extensor bursts (cf. the 1st and the 4th IP and TA bursts in Fig. 7A). They may represent abortive flexor bursts depressed somehow by the mechanism responsible for the prolongation of extensor burst.

## DISCUSSION

The spinal cord can no doubt coordinate the hindlimbs in two different modes in alternation during walk or trot or in non-alternation as in a gallop (Fig. Forsberg & Grillner 1973 cf. Müller & van der Meché 1976). The switch between them is sudden and not gradual. It may occur already at low speeds but the likelihood increases at high speeds. There is, however, no necessary link between speed and type of coordination as an animal may sometimes switch at very low speed and some minutes later only at a higher. Either the velocity or duration of the extension or the duration of the support phase can probably be a critical factor determining the switch between the different gaits. However, they can thus not be the only factors.

That the two legs are controlled by two separate central spinal networks, one for each limb (on Holst 1939; Shik & Orlovsky 1965; Kalugin & Shik 1970; Grillner & Zangger 1979) is a view that has been further corroborated in the present study as one limb could be made to walk one step when the other performed two or even four steps (Figs 7 and 8). Clearly this shift could be achieved by spinal

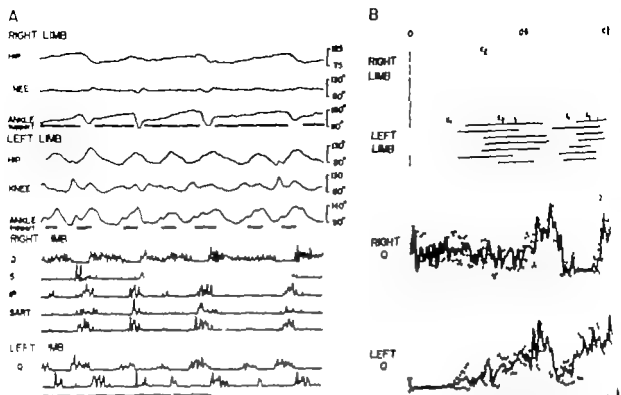


Fig. 7 Relative coordination (1) during split belt locomotion. A The angular movement in hip, knee and ankle together with the period of support (bars) for the right and left limb are shown at the top and the simultaneously recorded EMGs (rectified and filtered) are presented below. The velocity of the right belt varied between 0.88 and 0.90 m/s and that of the left was 0.90 m/s. Detail as in Fig. 3. Note time and angular calibration. B Schematic representation of the movements normalized to the stepcycles is shown at the top and the normalized and averaged Q-spectra shown below during a stable 2:1 rhythm. The support phase ( $E_1 + E_2$ ) is indicated with a bar (the swing phase subdivided in the flexion (F) and first extension (E) phase with a dot (determined from the angular movement at ankle). The rectified and filtered EMGs of the right and left Q (except for the second and sixth right stepcycle) averaged after normalization. The mean is indicated as a continuous line and the standard deviation as dotted lines. Cycle starts with the onset of the support of the right limb. (From Lutz.)

ences periods of stable 1:1 coordination occurred (Fig. 7B) and with larger differences 3:1 and even 4:1 rhythms could be obtained (Fig. 8).

The first extension phases and the onset of extension activity of the two limbs never appeared to overlap (see Figs. 7A, B and 8). If one limb approached ground to make contact, the other waited (if in this part of the stepcycle) until the contact and proceeded only thereafter with its own F. The support phase of the "fast limb" was often completed before the "slow limb" made contact (Fig. 7B; see legend Fig. 8). During one stepcycle of the slow limb, there was always a multiple of steps in the fast limb. This was probably due to the fact that the "fast limb" executed one step in relation to the swing phase of the slow limb and 1 to 3 steps during the support phase.

The duration and the structure of the stepcycles of the "fast limb" differed markedly depending on

the phase of activity in the other limb. During stable 2:1 rhythm the steps occurring during the contralateral swing phase were much shorter than during the support phase, with a reduction of the elements of the cycle (Fig. 7B). At higher rhythms (i.e. 3:1 and 4:1) the overall frequency of the fast limb was higher than during treadmill locomotion at the same speed. The stepcycles that occurred during the swing phase (flexion with flexion at the very end of the swing phase) was the shortest as during 1:1 rhythm (Fig. 8). The shortening was due to a short and prompt F<sub>1</sub> while the support phase was relatively long (Fig. 8; see ankle). In other parts of the contralateral stepcycle (i.e. the support phase) the flexion was slower, the first extension phase longer and the support phase relatively shorter.

#### 4 Reciprocal action during slow walking

(1), the "fast limb" performs shorter steps than during normal conditions. In spinal walk, as one limb could be stopped with maintained activity, while the other continued to walk at a higher rate (Grüner & Rossignol 1978; Grüner & Zangger 1979). A position does not crossed effect on the rhythm does also under these conditions. If the stopped limb is in a flexed position the walking movement on contralateral side will be rather feeble, but if the is extended to a point close to that in which it normally is flexed, the walking move-

ment becomes vigorous (Forsberg, Grüner & Rossignol unpubl.). The duration of the support is during locomotion on split belt as well as amplitude of the leg movement are almost the same as that, which would be observed in walking with "tied belts". At extreme situations (e.g. at the transition to 1) with strict relation be modified and the amplitude of the leg movements changed. These results clearly allow conclusion that the duration of the support is controlled not exclusively but to a very small degree by peripheral signals (Pearson & Scott 1976, Grüner & Rossignol 1978; Annett et al. 1978) from the moving limb. The neural input from each limb thus tends to separate the rhythms of two limbs that are not walking at the same speed. There occur however compensatory changes of the stepcycles that at moderate differences lock the limbs in a common beat. On the "fast side" the first part of the cycle is comparatively unchanged but a prolongation occurs in late F or in the first half of E<sub>1</sub>. The delay even occasionally "waits" at the transition near E<sub>1</sub> and E<sub>2</sub>. On the "slow side" there is really a shortening of the flexion phase and sometimes also a reduction of the support phase. This latter effect is however opposite to the peripheral influence on the support phase. The steps therefore preferentially occur during the swing phase and then especially in the interval prior to placement. After an unpredicted disturbance in movement in one limb which elicited a compensatory reaction that prolonged or reduced the duration of the stepcycle, a regular walk was still established by readjustments in the following swing phase (Forsberg et al. 1977 cf. Forsberg 1979). In long cats with one limb partly denervated and recorded EMG recording, substantial readjustments also occur during the extensor bursts

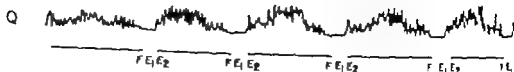
(Duyssens 1977). In this situation however the phasic peripheral feedback was severely disturbed (see however also Duyssens & Stein 1978). Hence the duration of the different phases are all modifiable at a given stepcycle duration. The structure of the stepcycle is thus highly modifiable. This is at variance with Kulagina & Shik (1970) who concluded that the structure of the stepcycle was determined by the speed of the belt and kept constant also during the split belt situation in contrast to the duration of the whole stepcycle which was allowed to vary. They did actually encounter changes of 10 and sometimes up to 30% in their material. They measured however the extension and flexion of the hip which may have rendered the disclosure of these effects difficult.

The fact that the different part of the stepcycle are modifiable becomes even clearer when one considers the 1 (or 3:1) coordination when the two steps on the split belt are asymmetric despite the fact that the belt speed is the same. This can in fact be seen also in one illustration of Kulagina & Shik (1970: Fig. 3) in which the step of the "fast limb" occurring during the swing phase of the "slow" has a faster flexion compared to steps occurring at the support phases of the slow limb. Thus the structure of a step depends on the prevailing phase of locomotor activity in the other limb. Hence crossed signals do not only control the coordination between the two sides but may actually also influence the cycle duration, the duration of the different phases, i.e. the structure of the cycle as well as the step length on the contralateral side.

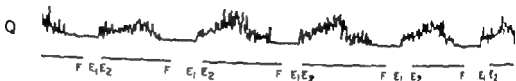
*Interlimb coordination.* The coordination between the two limbs is not achieved by a fixed lag between two separate events in the respective stepcycles (see Result sect. II 2). It is more likely that central signals related to the muscle activity in one or several phases of the step on one side project to the other side too. These signals may take form of reference copies (Stein 1974 and 1976 see Introduction). Undoubtedly interaction of some sort occurs between the different elements of the two central generator networks at the spinal level (Grüner & Zangger 1979). As the basic design of the neuronal network that constitutes the generator is unknown it is impossible at present to hypothesize about what type of neuronal interactions might occur between the two generators. On the other hand it is possible to define how the two networks actually do interact.

A 0.07 m/s

right



left



B 0.70 m/s

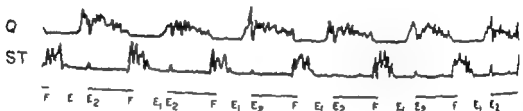


Fig. 9 Reciprocal actions. A The rectified and filtered EMGs of the right and left Q during very slow walking are shown together with the schematic movements of the ankle. The bars indicate the support phase, the dots the onset of the extension phases (E). B The rectified and filtered EMGs of Q and St of the same limb are shown together with schematic movements of the ankle. Note differences in time calibration. (From Åkima.)

mechanisms without influence from supraspinal structures.

The neuronal mechanism allowing the switch between "gallop" and alternate modes of coordination has been depicted as depending on two alternative pathways. One might provide reciprocal inhibition between congruent parts of the two generators, e.g. the flexor parts could mutually inhibit each other resulting in alternation (a similar effect would be achieved by excitation between extensors on one side and flexors on the other). Another might mediate mutual excitation between corresponding elements of the two generators and thereby elicit the gallop type of coordination (see Halbertsma et al. 1976; Grillner 1975). A control signal (e.g. descending or reflex) that caused a switch from one alternative pathway to the other could automatically switch the gait from one type to the other in a very simple way. This general idea has so far not been tested experimentally.

### The split belt situation

**Effects in the stepcycle.** A readjustment of stepcycles of the two sides are necessary when an animal walks along a curvature. The experimenter with split belts mimick this situation and show that spinal mechanisms are sufficient to induce the appropriate changes. Moreover, in the experimental situation the differences can be taken much larger than during ordinary conditions and thereby are properties of the spinal coordinating system as well as of the organization of the locomotor generator.

In decerebrate cats the "slow" and the "fast" limb will at moderate speed differences have cycle duration that is intermediate between that of walking at either the slow or the fast speed (Åkima & Shik 1970). This may be true also in the squirrel (see Table 1). That means that the activity in the fast limb influences the locomotor activity in the slow limb. At different rhythms

11, the "fast limb" performs shorter steps than during normal conditions. In spinal walk as one limb could be stopped with maintained activity while the other continued to walk at a higher rate (Griffner & Rossignol 1978; Grillaer & Zangger 1979). A position-dependent crossed effect on the rhythm does also under these conditions. If the stopped limb is at a fixed position the walking movements on the contralateral side will be rather feeble, but if the limb is extended to a point close to that in which a normally initiated walking movement will become vigorous (Forsberg, Griffner & Grillner, unpubl.). The duration of the support during locomotion on "split belts" as well as the amplitude of the leg movements are almost the same as those which would be observed during walking with "tied belt". At extreme situations (e.g. at the transition to 2:1) this strict relation is modified and the amplitude of the leg movements changed. These results clearly allow the conclusion that the duration of the support is controlled not exclusively but to a very high degree by peripheral signals (Pearson & Aert 1976; Griffner & Rossignol 1978; Aert et al. 1978) from the moving limb. The lateral input from each limb thus tends to separate the rhythms of two limbs that are not walking at the same speed. There occur however compensatory changes of the stepcycles that at moderate differences lock the limbs in a common rhythm. On the "fast side" the first part of the support is comparatively unchanged but a prolongation occurs in late F or in the first half of  $E_2$ . The support may even occasionally "wait" at the transition between  $E_1$  and  $E_2$ . On the "slow side" there occurs a shortening of the flexion phase and sometimes also a reduction of the support phase. This latter effect is however opposite to the peripheral influence on the support phase. The support therefore preferentially occurs during the flexion phase and then especially in the interval prior to placement. After an unpredicted disturbance in movement in one limb which elicited a compensatory reaction that prolonged or reduced the duration of the stepcycle, a regular walk was still established by readjustments in the following swing phase (Forsberg et al. 1977; Forsberg 1979). In walking cats, with one limb partly denervated and prepared for recording, substantial readjustments could also occur during the extensor bursts

(Duysens 1977). In this situation, however, the phasic peripheral feedback was severely disturbed (see however also Duysens & Stein 1978). Hence the duration of the different phases are all modifiable at a given stepcycle duration. The structure of the stepcycle is thus highly modifiable. This is at variance with Kulagin & Shik (1970) who concluded that the structure of the stepcycle was determined by the speed of the belt and kept constant also during the split belt situation in contrast to the duration of the whole stepcycle which was allowed to vary. They did actually encounter changes of 10 and sometimes up to 30% in their material. They measured however the extension and flexion of the hip which may have rendered the disclosure of these effects difficult.

The fact that the different parts of the stepcycle are modifiable becomes even clearer when one considers the 1 (or 3:1) coordination when the two steps on the fast belt are asymmetric despite the fact that the belt speed is the same. This can in fact be seen also in one illustration of Kulagin & Shik (1970: Fig. 3) in which the step of the "fast limb" occurring during the swing phase of the "slow" has a faster flexion compared to steps occurring at the support phases of the "slow limb". Thus the structure of step depends on the prevailing phase of locomotor activity in the other limb. Hence crossed signals do not only control the coordination between the two sides but may actually also influence the cycle duration, the duration of the different phases, i.e. the structure of the cycle as well as the step length on the contralateral side.

**Interlimb coordination.** The coordination between the two limbs is not achieved by a fixed lag between two separate events in the respective stepcycles (see Result sect. 11.2). It is more likely that central signals related to the muscle activity in one or several phases of the step on one side project to the other side too. These signals may take form of efference copies (Stein 1974 and 1976; see Introduction). Undoubtedly interaction of some sort occurs between the different elements of the two central generator networks at the spinal level (Griffner & Zangger 1979). As the basic design of the neuronal network that constitutes the generator is unknown it is impossible at present to hypothesize about what type of neuronal interactions might occur between the two generators. On the other hand it is possible to define how the two networks actually do interact.

It is important to realize that any peripheral stimulus that affects the activity of one generator can indirectly via the coordinating links influence the pattern of activity of the generator of the other side. Crossed effects on the structure of the cycle have been demonstrated (Grillner & Zangger 1979) but it is not clear whether they are mediated in this indirect way or induced by direct crossed effects. The fact that central mechanisms for interlimb coordination exists does of course not exclude the possibility that crossed peripheral factors may also contribute during locomotion (see Forsberg 1979).

The coordination in the split belt situation is apparently not a random at any combination of speeds. In both 1:1 or 2:1 the two hindlimbs are coordinated in a seemingly stable way so stable even that the two stepcycles of the "fast limb" in a 2:1 gait are asymmetric in a characteristic way. It may be relevant that the best fit to strict alternation between the two limbs are the periods between onset of hip extension and foot contact which constitute the last part of  $E_1$  (see Fig. 6). Furthermore it is noteworthy that the occurrence of  $\square$  in one limb seems to exclude its occurrence in the other limb also at 2:1 or 3:4 gaits. The initial part of  $E_1$  may be passive in the knee and ankle but only after the termination of the flexor activity (ankle). In contrast the hip extension is actively controlled as well as other features including the early cocontraction of foot flexors and extensors (Engberg 1964; Forsberg et al. 1980). Adjustments of the step cycle may be made during this period ( $E_1$ ) (see above). For instance the ground contact must be smooth regardless of the quality of the terrain. This phase could also be involved in the coordinating control. Reciprocal inhibition between symmetrical events may thus be a possible contributor during alternate gaits. Other factors that may contribute could be a mutual net facilitation between neurones generating the same or different phases on the two sides. A facilitation from the neurones controlling flexion on one side (slow) to the flexion and extension net works on the other side (fast) could explain the interaction resulting in the quick but forceful step occurring during the swing phase of the "slow limb" (see Figs. 7 and 8).

It may be expected that not one but several different mechanisms are operating to ascertain the interlimb coordination. Each mechanism may "push or pull" to promote its particular coordinat-

ing feature. They will constitute at least two kinds of mechanisms: one for the gait mode and one for alternation (see above).

## CONCLUSION

The chronic spinal cat can coordinate its hindlimbs in a variety of ways not only in alternation and gallop modes but also in adjusting the relative duration of support and swing phase to meet specific requirements such as during rest. An interesting implication of the present work is that the brain does not necessarily need to coordinate itself with adjustments of the hindlimbs when the animal is turning. It would actually be sufficient if the front part to turn and the hindlimbs would be a spinal reflex machinery make the appropriate changes (cf. Andersson et al. 1978; Grillner 1979, 1980). A change-over from gallop to alternate might be achieved by a switch from one set of alternative spinal pathways to another. There is however no reason to exclude additional supervisory control mechanisms.

In the stepcycle of a limb the duration of the support phase is determined primarily by peripheral inflow. The first part of the flexion is rather constant whereas most of the necessary timing adjustments occur during  $F_1$  or the last part of the flexion phase. It must be emphasized however that no element in the stepcycle is constant—all is modifiable.

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# Release of gastrin and somatostatin into the gastric lumen of healthy subjects and patients with duodenal ulcer and achlorhydria

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WISÉN K, UVNÄS-WALLENSTEN S, EFENDIĆ S & JOHANSSON C. Release of gastrin and somatostatin into the gastric lumen of healthy subject and patient with duodenal ulcer and achlorhydria. *Acta Physiol Scand* 1980; 108: 797-800. Received July 1979. ISSN 0001-677. Gastrointestinal Unit, Department of Medicine, Department of Endocrinology, Karolinska Hospital and Department of Pharmacology, Karolinska Institute, Stockholm, Sweden.

Gastrin and somatostatin were measured in alkaline gastric aspirates in normal subjects, patient with duodenal ulcer disease in quiescent state and in patients with achlorhydria. Both peptides were released into the lumen. The gastrin-somatostatin ratio (G/S) in healthy subjects is approximately three. Duodenal ulcer patients had significantly lower G/S ratio due to lower gastrin and higher somatostatin levels whereas in patients with achlorhydria the G/S ratio did not differ from normal subjects.

and somatostatin have been demonstrated in G- and D-cells of the gastric antrum. These hormones are released into the portal vein as well as into the gastric lumen. Thus gastrin has been discovered in the gastric lumen in cat (Uvnäs-Wallensten et al. 1976) and dog (Rehfeld et al. 1978) and man (Johansson et al. 1978) and verified as gastrin 17 in the cat and as gastrin 17 and 34 (Rehfeld et al. 1978) in the dog. A pH-dependent release of gastrin and somatostatin into the gastric lumen of cats was shown following vagal stimulation (Uvnäs-Wallensten et al. 1977) with relative dominance of somatostatin during perfusion at pH 1 and of gastrin at pH 4-5. It has been suggested that the two peptides exert paracrine effects and interact locally to moderate gastric acid secretion from the parietal cells (Pearse et al. 1977). At pH 7.4 both gastrin and somatostatin are released into the perfusate probably due to a direct effect of the high pH on the mucosal cells (Uvnäs-Wallensten unpublished data). In the present study we have determined levels of gastrin and somatostatin in the gastric lumen in healthy subjects and in patients with duodenal ulcer disease and achlorhydria.

## METHODS

Gastrin and somatostatin were determined in gastric aspirates from 14 healthy subjects (21 to 34 years old, mean 24 years) without history of gastrointestinal disease, in 10 achlorhydric patients and in 10 patients with duodenal ulcer disease (28 to 54 years old, mean 44 years). The diagnosis of duodenal ulcer had been confirmed by X-ray and/or endoscopic examination. None had demonstrated complications such as perforation, massive hemorrhage or secondary gastric ulcer. On an average 11 years had elapsed since the first symptoms and the course of the disease had been relapsing. At the time of the present study no patient had symptoms or signs of an active ulcer. Achlorhydria had been established in 10 patients, aged 29 to 72 years (mean 58) by measuring the gastric secretory response to i.v. pentagastrin, 6 µg/kg (Pentaprin, JCI, England). The mean fasting serum gastrin level in the patient was  $293 \pm 86$  pg.

Informed consent was obtained from each subject and the study had been approved by the Ethical Committee of the Karolinska Hospital, Stockholm.

No sedation was allowed 24 h prior to the study. After an overnight fast the subjects were intubated with double lumen gastric tube composed of Ryle tube 14 FG (Portex, England) used for aspiration of gastric contents and another Ryle tube 10 FG ending 6 cm proximally to the first one, prepared with additional openings at the tip and used for instillation of sodium bicarbonate.

Table 1 Ratio between concentrations ( $\text{pmol l}^{-1}$ ) of gastrin and somatostatin in aspirates 1 and 2  
Mean  $\pm$  SE

	Sub- jects no	G/S ratio in aspirate 1 and		
		1	2	(1 + 2) <sup>a</sup>
Controls	14	3.7 $\pm$ 0.8	4.7 $\pm$ 0.7	3.0 $\pm$ 0.7
Duodenal ulcer	10	0.7 $\pm$ 0.3	0.6 $\pm$ 0.3	0.6 $\pm$ 0.3
Achlor- hydria	10	2.5 $\pm$ 0.6	1.8 $\pm$ 1.6	7 $\pm$ 1.1

<sup>a</sup>P-level < 0.02 for difference from controls

P-level < 0.01 for difference from controls.

With the subject on his left side, 50 ml isotonic sodium bicarbonate, pH 8 (Sodium bicarbonate 0.167 M, ACO Sweden) was instilled into the stomach during 2 min. A 170 ml sample of the gastric contents was withdrawn at 6 min after initiation of the instillation (aspirate 1) and was replaced by an identical volume of the solution. After another 8 min a second 170 ml sample was withdrawn (aspirate 2).

100 ml of each aspirate was immediately adjusted to pH 8 with 1 M-NaOH and boiled for 15 min. The sample thus reduced to 1/5 of the original volume was rapidly frozen and stored at  $-20^{\circ}\text{C}$  until analysed for hormone concentrations.

Concentrations of gastrin were determined by radioimmunoassay (Nilsson 1975) using antiserum 604 kindly supplied by professor J. Rehfeld, University of Aarhus, Denmark. Somatostatin was determined by radioimmunoassay using own antibodies (Esfendy et al 1978). Concentrations of gastrin and somatostatin were expressed in molar units in calculating ratios between hormone concentrations in the aspirates.

The remaining 70 ml of the aspirate was used for analyses of electrolyte concentrations. Concentrations of sodium were determined by flame photometry and of chlorides by coulometric titration. Concentrations of bicarbonate were determined after adjustment of the sam-

ple to pH 10 with NaOH. Backtitration was made with 0.03 M NaOH to endpoint pH 10 after addition of 0.1%  $\text{H}_2\text{SO}_4$  and boiling.

Wilcoxon's nonparametric rank test was used for significance. Results are given as mean  $\pm$  SE.

## RESULTS

The ratios between concentrations of gastrin and somatostatin in the aspirates were not different: the first compared to the second aspirate in either the groups studied. Healthy subjects drew 3.0  $\pm$  0.7 times more gastrin than somatostatin from the gastric lumen (Table 1). The G/S ratio was different in patients with achlorhydria but was significantly lower in duodenal ulcer patients ( $P < 0.01$ ).

The degree of volume reduction by boiling was similar in all series of aspirates (Table 2). Electrolyte concentrations in unboiled samples (Table 3) showed similar degrees of dilution and neutralization in the two control aspirates and the first aspirate from the duodenal ulcer group. Hormone concentrations as expressed in  $\text{pmol l}^{-1}$  in unboiled rates could thus be compared between healthy controls and first aspirate from patients with duodenal ulcer although values from all aspirates are given in Table 3. The low G/S ratio in the duodenal ulcer group was due to reduced levels of gastrin ( $P < 0.01$ ) and elevated levels of somatostatin ( $P < 0.05$ ).

## DISCUSSION

Gastrin and somatostatin were released into the lumen of the intact human stomach after instillation of a sodium bicarbonate solution. Healthy and

Table 2 Concentrations of electrolytes in unboiled aspirates from healthy controls ( $n=14$ ), patients with duodenal ulcer ( $n=10$ ) and achlorhydria ( $n=10$ )

Last paragraph shows that the volume reduction by boiling prior to hormone analyses was similar in all series.

Subjects	Asp	Na (mmol l <sup>-1</sup> )	Cl (mmol l <sup>-1</sup> )	$\text{HCO}_3^-$ (mmol l <sup>-1</sup> )	Volume reduction (%)
Controls	1	137 $\pm$ 7	2 $\pm$ 4	11 $\pm$ 8	80 $\pm$ 1
	2	139 $\pm$ 8	70 $\pm$ 3	177 $\pm$ 11	72 $\pm$ 4
Duodenal ulcer	1	139 $\pm$ 5	4 $\pm$ 4	117 $\pm$ 8	79 $\pm$ 1
	2	131 $\pm$ 7	4 $\pm$ 9	80 $\pm$ 14	82 $\pm$ 1
Achlorhydria	1	142 $\pm$ 10	8 $\pm$ 1	140 $\pm$ 7	80 $\pm$ 1
	2	154 $\pm$ 5	8 $\pm$ 1	119 $\pm$ 6	79 $\pm$ 1

### e3 Concentrations of gastrin and somatostatin expressed in pmol l<sup>-1</sup> in the duodenal aspirate

Concentrations are comparable only between control aspirates and first aspirate in the duodenal ulcer group. The degrees of dilution and neutralization differ from each other in the second aspirate from duodenal ulcer patients and both aspirates from patients with achlorhy-

Asp	Gastrin	Somatostatin
Control	15.4	7.1
1st aspirate	5.3	15.8
2nd aspirate	5.3	8.1
3rd aspirate	4.6	10.1
4th aspirate	10.5	13.3

n.d. < 0.05 for difference from control aspirates

average released 3 times more gastrin than somatostatin, and this was true also for achlorhy patients. On the other hand in the peptic ulcer patients the proportion between hormone levels was 1:1. The advantage of using an alkaline buffer in duodenal aspirates is that the action of gastric pepsin is prevented. However, proteolytic enzymes may be present in the aspirates from regular duodenal contents and have to be inactivated. By boiling the aspirates immediately upon arrival destruction of the hormones and artifacts in the radioimmunoassay were avoided (Wald et al 1976).

In comparison of hormone concentrations in the aspirates from healthy subjects and duodenal ulcer patients is justified because of the similar electrolyte concentrations in the aspirates indicating same degree of dilution and neutralization. The finding obtained was that reduced concentrations of gastrin and elevated concentrations of somatostatin contributed to the low G/S ratio in the duodenal patients.

The present finding of low gastrin concentrations in gastric aspirates in the ulcer patients is in line with the finding of decreased basal serum gastrin in ulcer patients (Korman et al 1971; Trudeau et al 1971). However, others have failed to show any significant difference in basal gastrin between serum in patients with duodenal ulcer and healthy controls (Walsh et al 1975). Polak et al (1976) found no

differences in the number of G- and D- cells between healthy subjects and the majority of duodenal ulcer patients.

According to Chayvaille the antral content of gastrin were not different in duodenal ulcer patients compared to healthy controls but the patients were found to have a 40% reduction of their somatostatin content (Chayvaille et al 1978). The apparent discrepancies between these data when compared to each other and to the present result might be due to the use of different procedures and assay. Furthermore, the number of hormone producing cells or the hormone contents in the antral wall may not necessarily reflect the hormone secreting capacity. In addition the mechanism by which the release of gastrin and somatostatin is stimulated by alkaline buffer has not yet been identified.

Another difference between studies is that the examined patients represent different stages of the peptic ulcer disease. Thus all patients examined by Chayvaille et al had symptoms of an active ulcer and an endoscopically verified duodenal ulcer with mild or marked inflammation of the antral mucosa. In contrast the present patients were examined in a symptom-free interval after a long history of uncomplicated relapsing duodenal ulcer disease.

Should the hypothesis of a paracrine interaction of antral gastrin and somatostatin (Pearse et al 1977) be correct, then the present data may well be interpreted in terms of a long standing adaptation resulting in a dominance of somatostatin over gastrin.

The possibility to recover gastrointestinal peptides from gastric and duodenal contents may provide a useful tool for studies of pathophysiological mechanisms in diseases of the gastrointestinal tract.

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# Functional properties of neurones in the posterior part of area 7 in awake monkey

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LEINONEN L. Functional properties of neurones in the posterior part of area 7 in awake monkey. *Acta Physiol Scand* 1980 108: 301-308. Received 13 July 1979. ISSN 0001-6777. Department of Physiology, University of Helsinki, Finland.

The posterolateral part of the parietal association cortex (area 7 of Brodmann) was investigated in three awake behaving macaque monkey using transdural microelectrode recording technique. Only one of the 114 cells isolated remained unidentified. Of the cells 79% responded to somesthetic (39%) or visual (23%) or both somesthetic and visual (17%) stimulation. The somesthetic receptive fields were large covering for instance the whole arm or hand. Most receptive fields are on the upper extremities. Half of the somesthetically drivable cells had bilateral receptive fields. The visually drivable cells responded only to stimuli moving in a certain direction. The effects of stimuli for most of the cells responding both to visual and somatosensory stimulation were touching of the skin and visual stimuli moving towards the cutaneous receptive field. Of the cells 20% were active only during the monkey's own movements, most often during grasping and manipulation with fingers. The results indicate that the area studied is specialized in the control of hand movements, e.g. grasping, grooming and somesthetic recognition of the form of objects.

**Key words:** Parietal association cortex, area 7, microelectrode recording.

In their pioneering study of cellular function in area 7 Hyvärinen & Poranen (1974) recorded neurones that were activated by visual and somesthetic stimuli. Mountcastle et al. (1975) and Lynch et al. (1977) also recorded the medial parts of area 7 and found that cellular activity in this area was mainly related to the control of eye movements, i.e. the cells were active only during the movements of the eyes or they responded to visual stimuli. Studies in the posterolateral part of area 7 showed that here cellular activity was related to the control of face and arm movements (Leinonen et al. 1979; Leinonen & Nyman 1979). The purpose of this study was to explore the functional properties of the posterolateral part of area 7 medial to the end of the Sylvian fissure which was not included in the previous studies.

Neurones investigated responded to visual and somesthetic stimuli or discharged only during the monkey's own movements. The activity of most neurones was related to the control of hand movements, e.g. grasping and manipulation. The results

of this study provide evidence for the concept that area 7 is intraregionally differentiated for the control of movements of different parts of the body.

## MATERIAL AND METHODS

Recordings were made in four hemispheres of two adult female and one juvenile male *Macaca speciosa*-monkeys weighing 6-8 kg. Experiments on each monkey lasted 2-8 weeks. During the daily experiments of 3-5 h the monkey sat in a primate chair with its head immobilized with a halo attached to the skull. The monkey could move its arms and legs. The arm movements observed during the recordings were usually triggered by offering a raisin to the monkey. The movements consisted of the following sequence: reaching, getting hold of and bringing the raisin to the mouth. In order to relate the cellular activity to the arm movements a sample device was constructed. This device, which showed that the monkey was reaching for was placed in front of the monkey. When the monkey's finger was within the distance of 0.5-1 cm from the raisin, photocell attached to the board above the raisin was activated. By observing the monkey's behaviour and using this apparatus we could determine whether the discharges of a

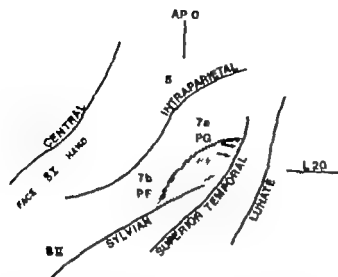


Fig. 1. The sulci of the parietal lobe and the recording site (hatched). The stereotaxic planes AP 0 and L 20 are indicated at the margins. The area recorded extends into the anterior wall of the superior temporal sulcus (arrows).

cell were related to (1) reaching and grasping for (2) manipulation or (3) bringing the rind to the mouth. Horizontal and vertical components of eye movements were recorded with electrodes implanted into the skull around the eyes. Cellular activity signals from ocular electrodes (timing signals of stimuli and verbal comment) were stored on tape. The method of recording has been described in detail earlier (Hyvönen & Poranen 1974; Leinonen et al. 1979).

During one penetration 8 neurones were usually isolated. After a neurone was examined the electrode was brought 1–2 mm deeper. Often the neurones had some "spontaneous" activity which revealed their existence. The spontaneous activity here means that the neurones discharged at a low irregular rate when the monkey looked at people moving and talking around him and when the monkey occasionally moved the eyes and limbs. Sometimes however only a certain stimulation or a certain motor activity evoked by the investigator revealed a new neurone. The cellular responses were observed on an oscilloscope screen. The cellular activity was also fed through an audio monitor to a loudspeaker; the discharges were thus audible all the time. We noticed regularly that a group of stimuli which were similar in spatio-temporal appearance or other information content could activate the same cell in varying degrees. Quantitative estimation of the differences was not however possible because of the nonstandardized stimuli. Because we did not use histogram technique only clear and strong responses could be observed and analyzed. The properties of each neurone isolated were studied by presenting different stimuli and observing the monkey behaviour. Following stimuli were used: light touch pressure on the skin, palpation and tapping of muscle bellies, rotation of joints, various moving and stationary visual stimuli (e.g. the experimenter's hand moving in different directions

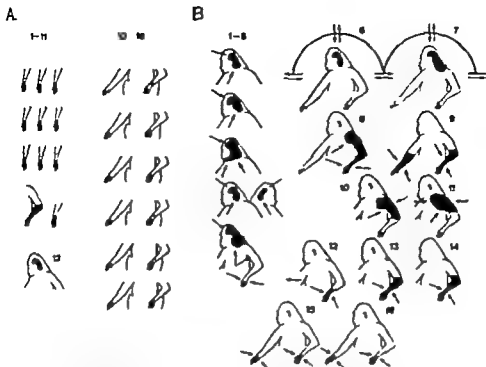
raisings bananas and novel small objects in front of the monkey).

All penetrations were made using the coordinate system of the Evans microdrive. A three-dimensional brain map of the target was obtained during recording. The target areas in two hemispheres of the two female monkeys were macroscopically and histologically investigated. After the monkey was killed several guiding electrodes were inserted into the target area. The locations of all penetrations were then studied in reference with the coordinates of these guiding electrodes. With the aid of each penetration could be located on the cortex. An accuracy of about 1 mm. Near the solid functional maps were also used for the identification of the location of penetration. These criteria included (1) distance of the neurone from the cortical surface (from the first histological activity recorded during the penetration) (2) distance of the white substance penetrated before reaching the cortex within a sulcus (3) location of the cell on the histological map obtained during the recordings. Using the marking electrodes the functional maps could be compared with the histological maps obtained from the brains anatomically examined in one monkey. In all still alive the penetrations were located using stereotaxic coordinates and functional criteria only. This method of localization of the target was considered to be sufficient for the purpose of this study.

The part of area 7 that was examined lies on the hemispheric surface and in the anterior wall of the superior temporal sulcus (Fig. 1). The superior temporal sulcus has been histologically examined by Selzer & Pandya (1972). They have defined four different subareas within the anterior wall areas PG, PGa, TAa and TPO. Area PG and 7a of Vogt & Vogt (1919) extend only a short distance into the sulcus. Most of our cells were isolated in the hemispheric surface and only a few neurones were found in the superior temporal sulcus.

Table 1. Classification of the cells isolated in the posterolateral part of area 7 according to the type of stimuli or type of behaviour related to the cell's activity.

	No. of cells	%
Cell responding to sensory stimuli	90	79
Somesthetic	44	49
— touch or pressure on the skin	18	20
— palpation of muscles or rotation of joint	26	29
Visual	26	29
Visual and somatic	20	22
Cell active only during the monkey's own movement	3	3
Reaching	1	1
Grasping and manipulation	2	2
Not identified	1	1
Total	114	100



2. Receptive fields of the cells which responded only to touching or compressing of the skin (A) and of the cells which responded both to touching (or compressing) of the skin and to visual stimuli (B). Arrows indicate directions of visual stimuli. Cells No. 6 and 7 responded to

## RESULTS

114 cells isolated and studied were classified according to their responsiveness to stimulation of somatosensory organs or according to the type of movement which correlated with the cellular activity (Table 1). Only one cell remained unidentified. Of the cells, 10% were active only during the monkey's own movements and 79% responded to some of the tactile, somatosensory or visual. The different classes of cells are reviewed below.

### Cells responding to touch or pressure of the skin

Seventeen cells responded only to cutaneous stimulation. Seventeen of them had a receptive field on the hand or wrist (Fig. 2A). Usually the cells responded in an on-off or on-off fashion to compression of the fingers or hands, only one cell was responsive to light touch on the skin and blowing into a hair. The cells that had a receptive field on the hand were active during the monkey's own hand

movement, especially during grooming when the monkey pulled a hair between the thumb and forefinger and/or when it released the hair.

### Cells responding to palpation from scales or rotation of joint

26 cells responded to palpation of a muscle belly in the arm (6 cells) or shoulder (7 cells) or they were active during rapid or forced dorsal flexion of the wrist, fingers (9 cells) or ankle (3 cells). Four cells responded to palpation of the calf. The cells that had a receptive field on the arm were active when the monkey was grasping for or manipulating an object with its hand. Fig. 3 presents properties of a cell which was activated by tapping and squeezing of the ulnar muscle bellies. The cell discharged also during the monkey's own movements. It had no spontaneous activity.

### Cells responding only to visual stimuli

Twenty-six cells were driven only by visual stimuli. They were all isolated near or in the anterior wall



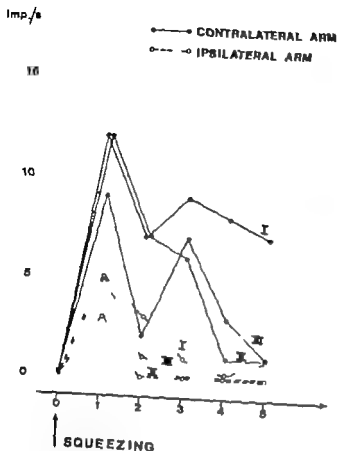


Fig. 3. Firing rate of a cell during the first 5 s of three successive squeezings of the ulnar muscle belly (I, II, III). Interstimulus interval 20 s. The responses disappeared in 5–10 s after beginning of the squeezing.

of the superior temporal sulcus. All these cells were activated only by moving stimuli. 10 cells by stimuli moving in a tangential direction upwards, downwards, left or right; 3 cells by rotating objects; 2 cells by stimuli moving from the periphery of the visual field toward the centre of it; 8 cells by objects that approached or were near the contralateral temple; 3 cells by objects moving near the contralateral side of the head.

Ten of the visually drivable cells were active when the stimulus moved in the periphery of the visual field. 15 cells responded to stimuli moving in the central visual field. The activity was not related to eye movements; this was examined by making the monkey gaze at something else during the presentation of the relevant stimulus or by analyzing from tape the temporal relationship between the cellular responses and eye movements.

#### *Cells responsive both to visual and somesthetic stimuli*

Sixteen cells responded to touching, pressing, stretching of the skin or palpation of a muscle as well as to visual stimuli approaching the somesthetic receptive field or moving in the periphery of the visual field (Fig. 2B). The receptive fields were on the hands or arms (8 cells), on the shoulder (4 cells) or on the temple (4 cells). Some cells were responsive to light touch on the skin and blowing on the hair; some only to compression of the skin which caused deformation. The cells that had a receptive field on the arm or hand responded to visual stimuli approaching the receptive field; the visual stimuli were effective in both halves of the visual field. Because of the head fixation the effective visual stimuli moving towards a receptive field on the shoulder always moved in the half of the visual field ipsilateral to the somesthetic receptive field. Fig. 4A shows responses of a cell to the investigator's hand approaching the contralateral temple (within 30 cm). During the withdrawal of the hand there is no activity. A stationary visual stimulus near the cutaneous receptive field resulted in intermittent activity. The activity stopped if the monkey's attention was drawn elsewhere with an interesting visual stimulus. In Fig. 4B the same cell responds, when the monkey's eyes are directed

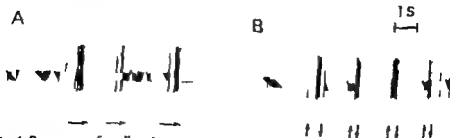
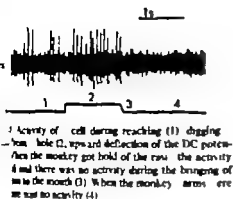


Fig. 4. Responses of a cell to the experimenter's hand approaching ( $\rightarrow$ ) the contralateral temple (A) and to touching ( $\uparrow$ ) of the temple when the monkey's eyes were closed (B).



activity of the contralateral temple. The effect of visual stimuli usually caused no noticeable contraction, not even the blinking reflex. The responses were not related to any degree of muscle contraction.

#### Discharging only during the monkey's own movements

Cells are active only during the monkey's own movements. 3 cells during reaching and 19 during reaching and manipulation with hands and one during manipulation with toes. All neurones except one activated by movements of both sides of the

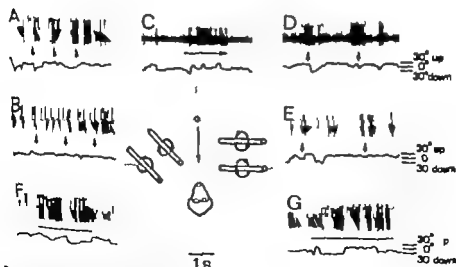
body. Fig. 4 shows discharges of a cell during reaching for and digging a ramrod from a hole. As soon as the monkey got hold of the ramrod the activity stopped although the flexion of the fingers still continued.

#### Cell discharging both during manipulation with fingers (as covered) and during visual stimulation

Two cells responded to object rotating in the visual field and were also active during manipulation (Fig. 6), two cells discharged when the monkey grasped an object with its eyes closed or when the monkey saw the investigator grasp an object (Fig. 7). The intensity of the response depended on the interest the object seemed to evoke in the monkey.

#### Laterality of the cells

One half of the cells isolated in area 7 responded to stimuli on either side of the body or either half of the visual field or they discharged during the monkey's own movements of either side of the body. Laterality of the effects of visual stimuli was tested by diverting the monkey's gaze to an other stimulus or by observing the direction of gaze during repetitive stimulations. The other half of the cells were activated only by stimuli on the contra-



4 Responses of cell to different visual stimuli. Signal from vertical eye movements under the cellular activity: lateral side of the monkey. C: the erect tube moving in the midsagittal plane towards the monkey. D and E: the erect tube in horizontal plane at eye level on the ipsilateral side of the monkey. F: monkey manipulates the tube with the eyes open. G: manipulation with the eyes covered.

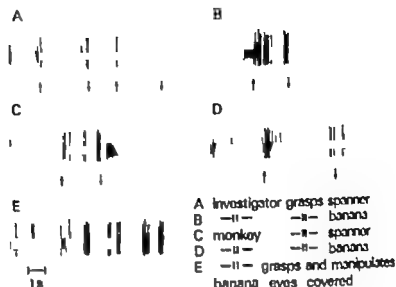


Fig. 7. Discharges of a cell in different conditions. Arrows in the figure indicate taking hold of an object with hand (↑) and releasing of the object (↓).

lateral side of the body (in reference to the hemisphere being recorded) or visual field or during movements of the contralateral side of the body (Table 2). No cells were activated only by ipsilateral stimuli. When a cell responded to somesthetic stimuli on both sides it usually responded more strongly to contralateral stimuli.

#### Referent body part

The functions of most cells which responded to somesthetic stimuli or were active only during the monkey's own movements were related to the sensory motor control of the arms and hands, i.e. they had a receptive field on the arm or hand or were active during grasping, manipulation or picking the fur with the fingers (Table 3). Twelve cells had their receptive fields on the head, 9 on the shoulder and 7 on the legs. No receptive fields were found on the trunk.

Table 2. Laterality of the receptive fields and body parts whose movements were related to the cellular activity in the posterolateral part of area 7.

	No. of cells	%
Contralateral	46	40
Ipsilateral	—	—
Bilateral	97	80
Total	113	100

#### DISCUSSION

##### Results compared with those obtained from neighbouring areas

The area examined is cytoarchitecturally part of area 7 of Brodmann (1909) and Vogt & Vogt (1919) and areas PF and PG of von Bonin & Bailey (1947). It borders medially and anteriorly on the cuneus, 7 and laterally on the parietal retromarginal sulcus within the Sylvian fissure and on the parietal opercular association area lateral to the Sylvian fissure. Functionally the area examined resembles the posterolateral part of area 7 which has been most extensively investigated (Leinonen et al. 1979; Leinonen & Nyman 1979) in the following respects: (1) the cells respond both to somatosensory and visual stimuli.

Table 3. Location of the somesthetic receptive fields and the body parts whose movements were related to the cellular activity in the posterolateral part of area 7.

	No. of cells	%
Arm and hand	95	80
Head	12	10
Shoulder	9	8
Legs	7	6
Shoulder and hip	1	1
Hand and feet	1	1
Total	113	100

the cell discharge only during the monkey movements (3) many cells have bilateral receptive fields or are active during movement of one side of the body. The functions of most cells in the lateral part of area 7 were related to control of face and arm movement e.g. reaching the lips or arm, bringing an object to the mouth. These functions in the area described in this work were mainly related to the control of movements i.e. grasping and manipulation of objects in the neighbouring parietal retroinular cortex (area 7). Pandya & Sanbales (1973) respond to somesthetic stimulation, most of them only to stimulation of the skin in on, off or on-off (Lemon 1979). In the present study some cells responded in a similar fashion to similar stimulation. The functional resemblance may be due to projection from area 7 to the area examined. Some properties of the cells in the neighbouring parietal association cortex (area 7) include responsiveness to somesthetic stimuli on the temple, shoulder or responsiveness to auditory stimuli some cells are only during the monkey's own head movements (Lemon et al. in preparation). Cells from area 7 suggest that it participates in control of head movements. In the present study some cells in the transitional region between area 7 and Tpt were activated by touching of the temple and visual stimuli approaching the temple. In a natural condition would trigger turning of the head towards the stimulus. Thus it can be assumed that these cells are part of the system controlling the head movements.

#### *Active only during the monkey movements*

Cells active only during the monkey's own movements have been reported in several works conducted in area 7 (Hyvarinen & Poranen 1974; Leinonen et al. 1975; Lynch et al. 1977; Leinonen et al. 1979; Leinonen & Nyman 1979). It has never yet been settled whether these cells respond to stimuli which cannot be produced actively (2) are functionally dependent on the activity of the motor and premotor cortex (collaboratory large gaiting phenomenon) (3) participate in analysis of the information content of a stimulus and in setting a goal for an action, (4) connections of the motor system. Area 7 has several connections with areas where some or all cells

discharge only during active movement. (1) motor areas arm region in the primary motor cortex send afferents to the lateral part of area 7 (Pandya & Kuypers 1969). (2) Frontal association area area 7 project to the prefrontal cortex (Chavira & Pandya 1976) and receives afferents from there (Stanton et al. 1977). (3) the ventrolateral nucleus of the thalamus efferents from area 7 have been described by Kaas et al. (1977) afferent to area 7 by Balesdier & Maugure (1977) and Stanton et al. (1977). The several anatomic connections with the motor control system indicate that the function in the parietal association cortex depend on motor activity. On the other hand some pyramidal tract fibers originate from neurones of area 7 (Peele 1944) which suggests that the functions of area 7 are necessary for some movements. Examination of these problem was not possible with the methods of the present study.

#### *Intersensory association*

Many cells responded to touching of the hand (when the monkey's eyes were covered) and to visual stimuli approaching the arm or hand. These cells were usually active also when the monkey himself was reaching for or manipulating an object. Apparently these cellular functions were related to the analysis of the direction, location and distance of a moving visual stimulus in reference to the body. Thus it can be suggested that the area examined participates in the spatial control of hand movements in the somesthetic and visual spaces.

It has been shown that monkeys succeed in cross-modal recognition (visual-tactile) of objects (Covey & Weiskrantz 1973; Jarvis & Ertling 1977; Bolster 1978). Some findings of the present study suggest that area 7 may participate also in cross-modal recognition of manipulable objects. Some cells were active when the monkey manipulated an object with its eyes covered and also when an object was rotating in the visual field.

#### *Result in the light of ablation studies and electric stimulation experiments*

Ablation of the parietal association cortex of the monkey result in deterioration of several different functions. Some of these functions were observed to be related to cellular activity of the part of area 7 examined. Somesthetically guided finger movements and tactual recognition of food (Peele 1944) opposition of the thumb and forefinger during grasp-

ing (Faugier-Grimaud et al 1978) tactile shape discrimination (Ruch et al 1938 Bates & Ettlinger 1960 Ettlinger & Kalsbeck 1962). It could be suggested that a lesion in the area examined would result in deterioration of these functions.

Electric stimulation of different parts of the parietal association cortex causes various movements of the eyes and arms; stimulation of the cortex above the end of the Sylvian fissure produces movements of the hand (Vogt & Vogt 1919 Lilly 1958) which is in accordance with the results of the present study.

The technical assistance of Ritva Kettunen, Tuula Nikkinen and Ilkka Linnankoski is gratefully acknowledged. I also thank Prof. Juhani Hyvärinen for the support he gave during the work and for critical comments on the manuscript. This work was supported by grants from the Academy of Finland, Research Council for Medical Sciences and from Emil Aaltonen Foundation.

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# respiratory effects elicited in newborn animals by the central chemoreceptors

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Central chemoreceptors on the ventral surface of the medulla oblongata play an important role in respiratory control in the adult. Increased  $\text{CO}_2$  tension in the blood leads to an increase in the cerebrospinal fluid (CSF)  $\text{H}^+$  concentration, exciting the central chemoreceptors leading to an increased respiratory drive and vice versa (for review, see e.g. Schick 1979). The present experiments aimed at studying the effects on respiration that can be elicited via the central chemoreceptors in the newborn animal and thus get a first insight into their importance for the establishment and maintenance of a normal and regular respiration in the newborn. Apnoeic breathing is not seldom seen in newborn animals, especially those born pre-term (e.g. Horowitz 1978) and is sometimes a forerunner to asphyxia. Increased  $\text{O}_2$  tension as well as increased  $\text{CO}_2$  tension counteracts periodic breathing. Recently Chernick et al. (1979) have demonstrated in the adult cat that local cooling of the medullary areas on the ventral medulla surface, i.e. the afferent fibres from the adjacent rostral central chemoreceptor areas have been suggested to converge, potentiates periodic breathing. One aim of the present experiments was to investigate whether periodic breathing can be produced in the newborn animal by inhibiting the central chemoreceptors.

Experiments have been performed on newborn rabbits, extremely immature at birth, and on newborn guinea-pigs, mature at birth. The rabbits were anaesthetized with urethane (usually 1000 mg/kg b.wt.) and additional doses given, as needed, the guinea-pigs with a solution of chloralose (25 mg/kg b.wt. i.p.) and urethane (1 mg/kg b.wt. i.p.). The animals were tracheotomized and spontaneous breathing. The trachea, esophagus and cervical muscles were divided and the frontal aspect of the aortic arches exposed. The aortic-occipital sulcus was carefully separated from the dura and the dura was incised through the dura and arachnoid. A fine catheter (1 mm) was inserted through this hole with the tip placed

just over the chemosensitive zones. In this catheter the chemoreceptor zones could be superfused with mock CSF of different pH or with GABA (0.1 or 1 mg/ml) dissolved in mock CSF. In some experimental near-term guinea-pig fetuses were exteriorized and the above preparation performed with the umbilical cord circulation still intact and before the breathing had been established. Mock cerebrospinal fluid were prepared according to Mitchell et al. (1963) and bubbled with  $\text{CO}_2$  to desired pH values. Respiration was recorded as thorax movement to the abdominal skin just below the sternum and connected to a force-displacement transducer writing on a Grass polygraph. In some of the experiments arterial blood pressure and heart rate were monitored in a catheter cannulating the left carotid artery. Periodic breathing was defined as respiratory pause of 5 s or more followed by breathing period of 20 s or less with the cycle repeated at least three times during 1 min.

The central chemoreceptors were found well-functioning from the first day of life both in the rabbits and the guinea-pigs. Pronounced respiratory changes were elicited ranging from strong augmentation of the ventilation when superfusing the central chemoreceptors with acid CSF to inhibition of the respiration and even apnoea with alkaline CSF (Fig. 1). Decreases in heart rate and blood pressure accompanied the inhibitory effects on respiration. Application of GABA elicited prompt inhibition of tidal volume and respiratory rate accompanied by decreased heart rate and blood pressure fall (cf. Wennergren & Öberg 1980) and easily induced apnoea. In the exteriorized guinea-pig fetuses clear cut respiratory effects could be induced via the central chemoreceptors immediately after the cord had been clamped and air breathing movements initiated (cf. Herrington et al. 1971).

Fig. 2 demonstrates how inhibition of the central chemoreceptors leads to periodic breathing. When the inhibition is relieved the respiration becomes regular again (top panel). When the sequence is repeated with increased  $\text{FIO}_2$  the inhibition of the

ing (Faugier-Grimaud et al 1978) tactile shape discrimination (Ruch et al 1938 Bates & Ettlinger 1960 Ettlinger & Kalsbeck 1962) It could be suggested that a lesion in the area examined would result in deterioration of these functions

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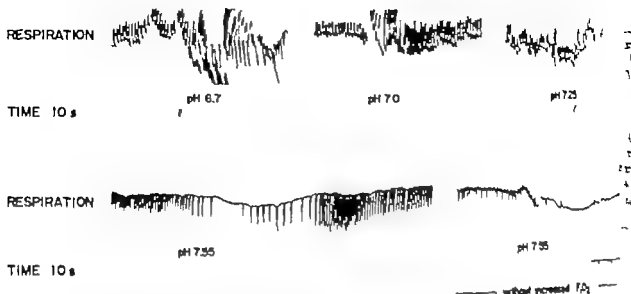


Fig. 1 Recordings of respiration in a newborn rabbit of a few h age. Note pronounced excitation of the respiration by superfusing the central chemoreceptors with acid CSF and inhibition with alkaline CSF. In the last sequence the rabbit breathes room air. In the preceding ones 40% oxygen.

central chemoreceptors only leads to reduction of the ventilation but the regular pattern is maintained (middle panel). The same response patterns were seen in newborn guinea pigs. Raising  $F_{iO_2}$  during

periodic breathing reversed the pattern to regular. Malfunctioning central chemoreceptors and a deficient respiratory drive from them may be a mechanism leading to periodic breathing also in the newborn infant. Interestingly a significant increased frequency and duration of periods of breathing during sleep recently was reported in rats who had had near-miss sudden death syndromes (Kelly & Shannon 1979). The authors discussed malfunction at the central chemoreceptor level as one possible explanation of their findings.

In conclusion the present experiments demonstrate well-functioning central chemoreceptors from the first day of life in both rabbits and guinea pigs. Further study is needed to elucidate what part the central chemoreceptors play in the establishment of a regular respiration at birth. Experiments on these lines are in progress. It is possible to produce periodic breathing in these newborns and by inhibiting the central chemoreceptors supports that impaired respiratory drive from the central chemoreceptors may be one cause of periodic breathing also in the newborn infant. An animal model is here provided for the production and study of periodic breathing in the newborn.

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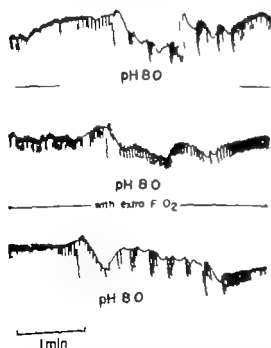


Fig. 2 Recordings of respiration in a rabbit age 1 week. Inhibition of the central chemoreceptors leads to the development of periodic respiration (top and bottom panel). When the inhibition is performed with the animal breathing 40% oxygen instead of room air a regular breathing pattern is maintained (middle panel).

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# Efflux of tryptophan and phenylalanine from bovine cerebral cortex synaptosomes

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As mediators of aromatic amino acids have been studied in detail only with brain slices (Ross et al. 1970; Črnčić et al. 1973; Laakso 1978; Oja & Korpi 1979a, b). An interpretation of data from slice work is complicated by the morphological complexity and cellular heterogeneity of the tissue. A specific transport mechanism has been proposed for L-tryptophan in synaptosomes (Dent & Sourkes 1977; Mandell & Kaapp 1977), but it has never been characterized in relation to transport of, for instance, other aromatic amino acids. A characterization of the efflux and exchange of tryptophan and phenylalanine has now been achieved in isolated superfused bovine brain synaptosomes.

Synaptosomes were isolated and purified from bovine cerebral cortices by ultracentrifugation across gradients as described by Kontro & Oja (1978). All subsequent experiments were carried out on the same day as the isolation of synaptosomes. The synaptosome fractions were metabolically viable and consumed oxygen in Warburg flasks at least 2 h at a constant rate of  $0.48 \pm 0.05$  mmol  $\pm$  S.E.  $\times$  5) mmol/s  $\times$  kg protein. Synaptosome samples for electron microscopy were prepared as described (Kontro & Oja 1978). The synaptosome fractions were fairly homogeneous, consisting mainly of torn-off nerve ending particles which synaptic vesicles and mitochondria could easily be identified (Fig. 1). Synaptosome suspensions (2 g protein/l) were deaerated for 15 min in Krebs-Ringer HEPES-glucose medium buffered to pH 7.4 at 310 K under  $O_2$  (5.5 mmHg) (0.05 mmol/l) L-[3- $^3H$ ]tryptophan or L-[3- $^3H$ ]phenylalanine. Synaptosomal particles (0.5 ml portions of the suspension) were layered on Millipore filters (pore size 0.5  $\mu$ m, Ø 3 mm) by vacuum. Another moistened filter was gently laid over the synaptosome layers and such assemblies were thereafter transferred into superfusion chambers built from Swinmax-25 (Millipore)

filter holders by reducing their internal volumes with plastered dental acrylic to 0.6 ml. Synaptosomes were first rinsed with 5 ml superfusion solution to remove extra synaptosomal label and superfusion experiments were thereafter carried out as described (Korpi & Oja 1979a). Semilogarithmic efflux curves constructed for [ $^3H$ ]tryptophan and [ $^3H$ ]phenylalanine were subjected to a graphical compartmental analysis (Solomon 1960) as depicted in Fig. 2. Two components were extracted from the total efflux.

Tryptophan was more effectively retained by synaptosomes than phenylalanine during 30-min superfusions with amino-acid-free media (Fig. 2). The half-times of the two efflux components were approximately the same for both amino acids (5 and 70 min) but the share of the slow component was three times larger in tryptophan than in phenylalanine efflux. These two components could originate from different synaptosomal compartments, but a more plausible explanation is that they represent separate efflux processes. The phenylalanine molecule is less hydrophobic than the tryptophan molecule (Bigelow & Channon 1976) and this circumstance may underlie the faster loss of phenylalanine from synaptosomes. Phenylalanine release is faster than tryptophan release also in rat cerebral cortex slices (Laakso 1978).

The effects of exogenous unlabelled tryptophan, phenylalanine and histidine on [ $^3H$ ]tryptophan efflux are depicted in Fig. 3. Tryptophan efflux was stimulated significantly more from the extracellular side by these amino acids than phenylalanine efflux (Table 1). This indicates that intrasynaptosomal tryptophan is more accessible for exchange with extracellular amino acids than phenylalanine. Spontaneous efflux of phenylalanine may also be non-mediated to a greater extent than tryptophan efflux, as is also suggested by the larger share of the fast component in phenylalanine efflux (Fig. 2) and by the greater rate constants for spontaneous efflux

Table 1 Effects of tryptophan, phenylalanine and histidine on the efflux and exchange of [ $^3$ H]tryptophan and [ $^3$ H]phenylalanine in synaptosomes from bovine cerebral cortex

The results (means  $\pm$  S.E.) give the efflux rate constants for the superfusion intervals indicated from experiments similar to those depicted in Fig. 3. Student's *t* test was used to assess significance of differences from the controls.  $P < 0.05$   $P < 0.01$

Amino acid present (1 mmol/l) from 18 to 22 min	No of expts	Efflux rate constants ( $\times 10^{-3}$ min $^{-1}$ ) for times stated		
		1-16 min	18-22 min	26-30 min
<i>Efflux of [<math>^3</math>H]tryptophan</i>				
None (control)	3	17.1 $\pm$ 0.9		
Tryptophan	4	15.5 $\pm$ 1.7	16.0 $\pm$ 0.9	1.3 $\pm$ 0.6
Phenylalanine	4		73.7 $\pm$ 5.5	11.2 $\pm$ 1.2
Histidine	4	17.6 $\pm$ 1.5	66.0 $\pm$ 5.4	11.8 $\pm$ 1.1
		15.4 $\pm$ 1.8	67.3 $\pm$ 8.3	9.5 $\pm$ 0.9*
<i>Efflux of [<math>^3</math>H]phenylalanine</i>				
None (control)	3	77.3 $\pm$ 1.2		
Tryptophan	4	26.1 $\pm$ 2.0	23.2 $\pm$ 1.5	11.8 $\pm$ 1.4
Phenylalanine	4		48.8 $\pm$ 7.2	20.5 $\pm$ 1.2*
Histidine	4	27.4 $\pm$ 2.6	48.2 $\pm$ 2.4	4.2 $\pm$ 4.2*
	4	26.0 $\pm$ 1.2	43.6 $\pm$ 1.8*	15.9 $\pm$ 2.1

(Table 1) The elevated efflux of phenylalanine still persisted when the medium was again amino-acid free while the efflux of tryptophan soon returned to the control levels. It is thus possible that phenyl-

alanine efflux was also enhanced by intrasynaptic mal amino acids whereas tryptophan efflux was influenced or even decreased in the case of histidine. In general no systematic significant differences

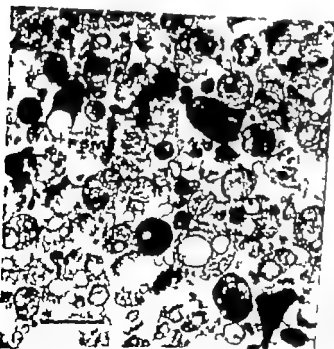


Fig. 1 Electron microscopy of bovine cerebral cortex synaptosomes. Synaptosomes surrounded by presynaptic membranes and containing synaptic vesicles, mitochondria and some vacuoles are visible. Electron opaque (arrows) and dense-cored (arrowheads) vesicles can be distinguished. There are some unidentified membrane profiles as contaminants. S, synaptosome PM; M, synaptic membrane; Mt, mitochondrion. Calibration bar 1  $\mu$ m.

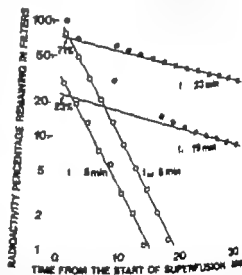


Fig. 2 Resolution of the efflux curves of [ $^3$ H]tryptophan and [ $^3$ H]phenylalanine into 2 components in synaptosomes from the bovine cerebral cortex. Synaptosomes were superfused with plain Krebs-Ringer HEPES plasma medium for 30 min. The percentage of [ $^3$ H]tryptophan (■) or [ $^3$ H]phenylalanine (●) remaining in synaptosome layers on Millipore filters is shown as a function of superfusion time in a semi-logarithmic plot. Each point is a mean of 5 expts. Relative S.E. less than 10%. Open symbols define the fast efflux component of [ $^3$ H]tryptophan (□) and [ $^3$ H]phenylalanine (○) efflux remaining after subtraction of the slow component from the total efflux. The percentage figures in the graph denote the shares of the fast efflux components. The *t* $_{1/2}$  values give the half times of the fast efflux components.



Fig. 1. Effects of extracellularly labelled amino acids on the efflux of  $[^3\text{H}]$ tryptophan from bovine cerebral cortex synaptosomes. Synaptosomes were preloaded and superfused as described except that at 17.5 min from the beginning the amino-acid free superfusion solution was exchanged for 4 min into the same solution supplemented with 1 mmol/l unlabelled tryptophan (O), phenylalanine (□) or histidine (●) or with the same un-supplemented solution (●). During this period there occurred exchange of extrasytoplasmic  $[^3\text{H}]$ tryptophan with unlabelled extrasytoplasmic amino acids. At the end of superfusion  $[^3\text{H}]$ tryptophan is released again into amino-acid solution together with the newly accumulated unlabelled amino acids. The graph gives representative results from 4 parallel experiments. The straight lines are computer regression lines for the data of the 3 various experimental phases (Korpi & Oja 1979b). The negative slopes of the lines are the efflux rate constants compiled in Table 1.

as could be demonstrated in the effects of tryptophan, phenylalanine and histidine on the efflux processes, although all efflux rate constants before and after superfusions with histidine tended to be numerically slightly smaller. Free accumulation of efflux by extrasytoplasmic amino acids scarcely demonstrates the participation of membrane transport sites in the stimulated efflux of tryptophan and phenylalanine. No fundamental differences in the efflux mechanisms of tryptophan and phenylalanine were revealed in the present

study. This circumstance does not corroborate the assumption of a transport mechanism specific for tryptophan in nerve endings. Our results from bovine cerebral cortex synaptosomes are apparently not at variance with the results of earlier studies on rat brain slices (Laakso 1978; Korpi & Oja 1979a, b).

The authors thank Mr J. Lankoniemi for his skilful assistance in making the superfusion chambers.

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Table 1 *Effects of tryptophan, phenylalanine and histidine on the efflux and exchange of [ $^3$ H]tryptophan and [ $^3$ H]phenylalanine in synaptosomes from bovine cerebral cortex*

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Histidine	4	15.4 $\pm$ 1.8	67.3 $\pm$ 8.3**	9.5 $\pm$ 0.9*
<i>Efflux of [<math>^3</math>H]phenylalanine</i>				
None (control)	3	27.3 $\pm$ 1.4	23.7 $\pm$ 1.5	11.8 $\pm$ 1.4
Tryptophan	4	26.1 $\pm$ 2.0	48.8 $\pm$ 2.2	20.5 $\pm$ 1.2*
Phenylalanine	4	77.4 $\pm$ 6	48.2 $\pm$ 2.4**	24.2 $\pm$ 4.2*
Histidine	4	26.0 $\pm$ 1.2	43.6 $\pm$ 1.8	15.9 $\pm$ 1

(Table 1) The elevated efflux of phenylalanine still persisted when the medium was again amino-acid free while the efflux of tryptophan soon returned to the control levels. It is thus possible that phenyl-

alanine efflux was also enhanced by intrasynaptosomal amino acids whereas tryptophan efflux was influenced or even decreased in the case of histidine. In general no systematic significant dif-

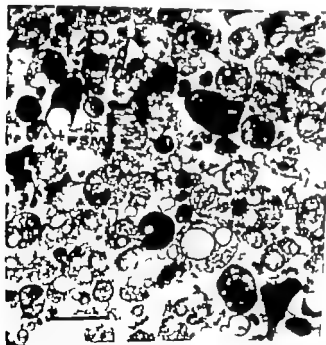


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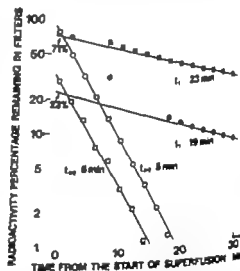


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## Production and evaluation of antibodies to glucagon albumin conjugates in the guinea pig

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Although various techniques have been reported for production of glucagon antisera, the stimulation of antibodies with suitable affinity and specificity for the radioimmunoassay of glucagon has been particularly difficult to achieve (Heding 1977). Inconsistent with the unique structural and physiological properties of guinea pig glucagon (Avalos & Tolobrotsky & Rowlett 1969; Sundby 1976), previous studies have underlined the particular value of the guinea pig for the rapid production of anti-glucagon antisera against glucagon-carbonyl-conjugates (Flatt & Swanston-Flatt 1979). Since there is evidence that the use of glutaraldehyde as the coupling agent results in the production of antisera with restricted specificity in the rabbit (Garaud et al. 1976), guinea pigs were immunized with glucagon-albumin conjugates prepared at an approximate molar ratio of 10:1 using either carbodiimide or glutaraldehyde as the coupling agent.

**Carbodiimide conjugation** 50 mg crystalline porcine glucagon (Calbiochem) was dissolved in 0.3 ml hydrochloric acid. To this was added 200 µg L-glutamine and 62.5 mg bovine plasma albumin in 5 ml water. The solution was made up to 18 ml with 300 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma) was added dropwise to 1 ml water. The mixture was stirred at room temperature for 24 h after which the preparation was extensively dialysed. The extent of coupling was approximately 80%.

**Glutaraldehyde conjugation** 10 mg crystalline porcine glucagon (Novo), 200 µg <sup>125</sup>I-glucagon and 2.5 mg bovine plasma albumin were mixed in 4 ml sodium phosphate buffer (0.2 M, pH 7.3). To this suspension was added 4 ml 5% glutaraldehyde. The mixture was stirred at room temperature for 24 h and then extensively dialysed. The extent of coupling was approximately 75%.

**Immunization** Groups of five male albino guinea pigs (150 g) were inoculated subcutaneously with either 500 µg glucagon (carbodiimide) or 300 µg glucagon (glutaraldehyde) emulsified in an equal volume of Freund's complete adjuvant. Secondary and subsequent injections were administered at adjacent sites every 4 weeks using the same doses of immunogen and incomplete adjuvant. The animals were bled by cardiac puncture 1-14 days after the second booster and all subsequent injections.

**Antibody assessment** The sera were assessed in terms of titre, affinity and specificity using a 450 µl equilibrium assay system dependent on 10 pg moniodinated porcine glucagon (Ungerwen & Larven 1977) and charcoal separation. Standard curves were prepared using a porcine glucagon standard (MRC London) and the specificities of the antisera were evaluated using a gut extract prepared from the entire mouse GI tract (Kenney 1955). This heterogeneous gut GLI preparation was calibrated using a fully cross reacting antiserum (R59) generously donated by Professor K. D. Buchanan.

**Results** As illustrated in Fig. 1 all guinea pigs immunized with the conjugate prepared using carbodiimide developed large amounts of high affinity glucagon antibodies which exhibited slightly different degrees of cross reaction with gut GLI. Antibody titre and affinity rose progressively during immunization attaining very high values by 22 weeks. In contrast the conjugate prepared using glutaraldehyde was markedly less effective in stimulating antibody production in the guinea pig. Thus of the 5 animals inoculated over a period of approximately 6 months, only one responded by producing glucagon antibodies (Fig. 1). The sera

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TITRE	AFFINITY	SPECIFICITY
8	75	3
8	—	—
4	50	2
2	25	1
—	—	—
0	0	0

Fig. 1 Characterization of glucagon antisera from guinea pigs immunized with glucagon-albumin conjugates prepared using carbodiimide (open circles) or glutaraldehyde (closed circles). Values for each animal are given as mean of 4 bleedings taken over a period of 22 weeks. The mean result for each group is indicated by the horizontal bar. Titre: log-inverse 50% maximum binding dilution. Affinity: % fall bound counts caused by 125 pg glucagon. 100 (Bo-B)/Bo. Specificity: ratio of tracer displacement caused by 125 pg glucagon and 125  $\mu$ g equiv. gut GLI.

derived from this particular animal contained antibodies of relatively high affinity which displayed partial discrimination between glucagon and gut GLI (Fig. 2). Antibody titres were slightly below average in this guinea pig and like affinity rose only gradually during immunization.

**Discussion.** Monthly immunization of guinea pigs with a glucagon-carbodiimide albumin conjugate was associated with the successful production of large amounts of high affinity glucagon antibodies. Indeed in a comparative study it has previously been suggested that the enhanced immunogenicity of such conjugates in the guinea pig makes this animal more attractive than the rabbit for the rapid production of potent glucagon antisera (Flatt & Swanson-Flatt 1979). However antisera raised in the guinea pig against the conjugate prepared using carbodiimide appear to invariably react almost equally well with glucagon and gut GLI. Thus although such potent antisera are particularly valuable for many studies they cannot justifiably be used for the assay of glucagon in plasma or other samples which contain additional GLI components.

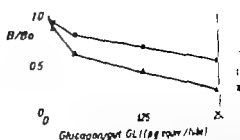


Fig. 2 Reaction of the sole responder in the pig group guinea pigs immunized with the glucagon-glutaraldehyde albumin conjugate (GPD4/3) with glucagon (squares) and gut GLI (circles).

The C-terminal portion of the glucagon molecule is believed to represent the specific immunodeterminant of antibodies which react poorly with gut GLI (Assan & Slusher 1977; Oheda et al. 1979). Accordingly glutaraldehyde which preferentially conjugates albumin to the amino groups at residues 1 and 12 of the N terminal fragment of glucagon has been reported to exclusively stimulate the production of antisera with restricted specificity in the rabbit (Garaud et al. 1976). Nevertheless it was not possible to achieve a similar degree of success with such a conjugate in the guinea pig. Indeed the use of a glucagon-glutaraldehyde-albumin conjugate in the present study was not associated with the production of particularly valuable glucagon antisera. The sole responder yielded high affinity antibodies which in accordance with the experience of Frohman et al. (1970) displayed partial discrimination between glucagon and gut GLI. Thus although the guinea pig represents a particularly valuable species for the rapid production of potent high affinity antisera against glucagon-carbodiimide protein conjugates antibody specificity remains a problem in this species.

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# apid simultaneous determination of regional blood flow and blood-brain glucose transfer in brain of rat<sup>1</sup>

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A new method was developed and used in rat to measure regional and whole-brain blood flow and blood-brain glucose transfer simultaneously and in 20 s. This simple method consisted of bolus injection of labeled butanol and tracer glucose, determination of the average arterial tracer concentration and subsequent assay of cerebral tissue activity 20 s after bolus injection. The whole-brain blood flow rate averaged 1.79 ml (100 g)<sup>-1</sup> min<sup>-1</sup>. The unidirectional blood-brain glucose transfer was twice as high as previously estimated in similar studies on rat, or 144  $\mu$ mol (100 g)<sup>-1</sup> min<sup>-1</sup> at 10 mM glucose in plasma. The magnitude is sufficient to explain the high cerebral glucose consumption rates recently determined by means of the autoradiographic 2-deoxy-D-glucose method of Sokoloff et al. (1977).

**Key words:** Regional cerebral blood flow, blood-brain glucose transfer, integral method.

Methods for determination of regional cerebral blood flow in the rat require determination of arterial, tissue, and/or cerebral venous indicator concentrations over a period of at least 60 s, and methods for determination of whole-brain blood flow require equilibration for at least 10 min. Length shortening of the time to 3 min has been recently achieved under conditions of very high blood flow rates.

In non-steady-states, procedures of shorter duration may be necessary. Such an approach is described below. The method is an extension of the integral method of Landau et al. (1955) and permits both brain and regional cerebral blood flow rates to be measured by a procedure lasting less than 10 s. In addition, we extended the method to involve simultaneous but independent determination of unidirectional blood-brain glucose transfer rates. D-glucose is the major nutrient of the brain and is transported from blood to brain tissue by facilitated diffusion. The unidirectional flux has never previously been measured rapidly under approximately steady-state conditions, and the response of blood-brain glucose transport to acute alterations of plasma glucose is therefore unknown.

In brief, the method is applied in the following way. A bolus injection of labeled butanol and tracer

glucose is given in a femoral vein. Arterial blood is slowly withdrawn into a syringe for 10 or 20 s from a femoral artery. At the end of the 10 or 20 s sampling is discontinued, the rat is decapitated and the brain removed, divided and deposited in closed vials for further processing.

## THEORY

### Determination of cerebral blood flow

The fundamental equation used to measure cerebral blood flow by means of indicator fractionation is the following,

$$F^B = \frac{C_{in}(T)}{E(T) \int_0^T C_p^B(t) dt} \quad (1)$$

in which  $F^B$  is the blood flow rate per unit weight of sample of brain,  $C_{in}(T)$  the indicator content per unit weight of brain tissue,  $E(T)$  the net extraction fraction of indicator in the time from introduction of the indicator into the circulation ( $t=0$ ) to the termination of the experiment ( $t=T$ ) and  $C_p^B(t)$  the arterial blood concentration of the indicator at the time  $t$ . The usefulness of equation (1) depends entirely on the knowledge of  $E(T)$ . The assumptions underlying the equation, its development and methods of estimation of  $E(T)$  will be presented below.

Presented in part at the 28th Annual Meeting of the American Academy of Neurology in Toronto, Canada, April 1976 (Schaefer et al. 1976).



Table 1. The cerebral extraction fraction of freely diffusible indicator in brain as a function of arterial extraction

n	Function	$E(T)$
1	$C_a^m(t)$	$e^{-p\left[\frac{f^m T}{\lambda_w}\right]} \int_0^T C_a^m(t) \exp\left[\frac{f^m t}{\lambda_w}\right] dt$ $\int_0^T C_a^m(t) dt$
2	$k$	$\frac{1 - e^{-p\left[\frac{f^m T}{\lambda_w}\right]}}{\left[\frac{f^m T}{\lambda_w}\right]}$
3	$k_1$	$\left[\frac{f^m T}{\lambda_w}\right] \left[ 1 - \frac{1 - e^{-p\left[\frac{f^m T}{\lambda_w}\right]}}{\left[\frac{f^m T}{\lambda_w}\right]} \right]$
4	$C_a^m(t') \exp[-k(t-t')], t > t'$	$\frac{k}{\left[\frac{f^m T}{\lambda_w} - k\right]} \left[ \frac{p\left[\frac{f^m(T-t')}{\lambda_w}\right] \exp\left[\frac{f^m(T-t')}{\lambda_w}\right]}{1 - \exp[-k(T-t')]} \right]$

is steady (i.e. to time  $T$ ) monoexponential decay from time  $t'$  i.e.  $C_a^m(t) = C_a^m(t') \exp[-k(t-t')]$  for  $t > t'$ , as shown in Table 1 for  $k$  and  $p$ .

$$\frac{\ln[p(T-t') - \exp[-p(T-t')]]}{(p-k)(1 - \exp[-k(T-t')])} \quad (9)$$

which  $p$  equals  $f^m/\lambda_w$ . However, the  $C_a^m(t)$  curve rises to a peak time  $t'$ . For  $E(T)$  calculated from equation (9) to be an acceptable approximation of the actual  $E(T)$  for a period 0 to  $T$  it must therefore be shown that the error of labeled blood in arterial blood prior to  $t'$  is not large.  $E(T)$  calculated by means of equation (9) is generally in error. It will be shown in Result that this is the case for  $T = 20$ . By insertion of equation (9) in equation (7) it is possible to express the brain content of indicator as a function of  $p$ ,  $k$ , and  $T$ .

$$\frac{\ln[p(T-t') - \exp[-p(T-t')]]}{(p-k)(1 - \exp[-k(T-t')])} = \frac{F^m C_a^m(T)}{\lambda_w Q_b(T)} \quad (10)$$

which equation (9) can be calculated when  $F^m$ ,  $C_a^m(T)$ ,  $\lambda_w$ ,  $Q_b(T)$ ,  $k$ ,  $T$  and  $t'$  are known, provided the assumptions underlying the equations (4) and (8) are reasonably fulfilled.

In the present study the equations were used as follows.

Initially  $k$  and  $t'$  were estimated from values of  $C_a^m(t)$ . Second  $p$  was estimated by fitting equation (9) to the experimentally determined values of  $E(T)$ . Third,  $f^m$  determined from equations (1) and (5) was compared to  $f^m$  calculated from equation (10) using also of  $\lambda_w$  obtained directly by equilibration of blood and brain tissue with labeled bromol.

Two problems interfere with the calculation of  $p$  (and hence  $f^m$ ) from equation (10): First the method as presented does not yield  $k$  and  $t'$  and second, equation (10) is transcendental equation which can be satisfied by one, two, or no positive values of  $p$ . The problems were addressed as follows.

In routine application of the method, the constants  $k$  and  $t'$  are estimated by the sample dependent of measuring the arterial indicator concentration  $C_a^m(T)$ , at the time of termination of the experiment. If

$$C_a^m(t) = C_a^m(t') \exp[-k(t-t')] \quad (t > t')$$

then

$$C_a^m(T) = C_a^m(t') \exp[-k(T-t')]$$

and by insertion of the expression for  $C_a^m(t)$  derived from these two equations into equation (6), with  $C_a(T)$  replaced by  $Q_b(T)$ ,  $f^m$  by  $F_b^m$  and  $E_b(T)$  by 1

Following introduction of an indicator into the circulation the indicator is taken up per unit weight of any organ including brain at a rate equal to the product of the blood flow rate per unit organ weight  $F^M$  and the arteriovenous deficit

$$\frac{dC_M(t)}{dt} = F^M (C_M^A(t) - C_M^V(t)) \quad (2)$$

where  $C_M^V(t)$  is the organ venous blood concentration of the indicator at the time  $t$  after introduction of the indicator into the circulation. When brain uptake of the indicator is entirely flow-limited i.e. equilibrium between tissue and venous concentrations occurs instantaneously the rate of change of brain indicator content can be expressed by the following modification (Kety 1951) of equation (2)

$$\frac{dC_M(t)}{dt} = F^M \left( C_M^A(t) - \frac{C_M(t)}{\lambda_M} \right) \quad (3)$$

in which  $\lambda_M$  is the tissue-blood partition coefficient of the indicator (ml/g). Provided  $F^M$  is constant and the tissue is homogeneous with respect to  $F^M$  and  $\lambda_M$ , equation (3) can be integrated to yield

$$C_M(T) = F^M \exp \left[ -F^M \frac{T}{\lambda_M} \right] \int_0^T C_M^A(t) \exp \left[ F^M \frac{t}{\lambda_M} \right] dt \quad (4)$$

Equation (4) was used by Landau et al. (1955) to measure regional cerebral blood flow in the cat 60 s after an *i.v.* bolus injection of antipyrine. In this case tissue concentrations were measured autoradiographically as was  $\lambda_M$  in regions of brain sufficiently small to allow the authors to argue that the conditions of integration were fulfilled. In the present study  $\lambda_M$  and  $F^M$  will be measured in regions of brain approximately corresponding to major subdivisions of the rat brain (cerebellar hemispheres, vermis, pons, midbrain, basal ganglia, parietal cortex and frontal, occipital and temporal lobes). These regions consist chiefly of grey matter for which it has been shown that oxidative metabolism and by inference  $F^M$  does not vary much within each region (Sokoloff et al. 1977).

$E(T)$ , the net extraction fraction of indicator in the zero to  $T$  time interval is defined by the following equation

$$E(T) = 1 - \frac{\int_0^T C_M^A(t) dt}{\int_0^T C_M^V(t) dt} \quad (5)$$

Insertion of the numerator from equation (5) in equation (2) after integration readily yield the fundamental equation (1). Equation (1) applies to any organ as well as to the whole body provided  $F^M$  is constant. Thus for a unit weight of another organ indicated by subscript  $o$

$$C_o(T) = F^M E_o(T) \int_0^T C_M^A(t) dt \quad (6)$$

If  $C_M^A(t)$  for the two organs are identical for all values of  $t$  from 0 to  $T$

$$F^M = F^M \frac{E_o(T)}{E(T)} \frac{C_o(T)}{C_M(T)}$$

In the present method, a reference organ is introduced in the form of a syringe into a heparinized artery at the rate  $F^M$ . In this case  $C_o(T)$  is substituted by  $Q(T)$ , the total radioactivity accumulated in the syringe at time 0 to  $T$  and  $E_o(T)$  equals 1. Thus for the relation between an organ and the syringe we have

$$F^M = F^M \frac{C_M(T)}{E(T)Q(T)}$$

Equation (7) is the operational form of the fundamental equation of the method. In whole-brain studies,  $E(T)$  be determined directly by equation (5) following analysis of arterial and mixed venous blood. For regional measurements in which  $C_M^A$  for each region is unknown,  $E(T)$  be determined by the following combination of equations (1) and (4)

$$E(T) = \frac{\exp \left[ -F^M \frac{T}{\lambda_M} \right] \int_0^T C_M^A(t) \exp \left[ F^M \frac{t}{\lambda_M} \right] dt}{\int_0^T C_M^A(t) dt}$$

which rests on the assumptions that underlie equation (1) i.e. homogeneity of  $F^M$  and  $\lambda_M$  in the region examined, equilibration of the flow indicator between tissue and venous blood and constancy of blood flow during the experiment.

Equation (8) can be solved numerically if sufficient numerous values of  $C_M^A(t)$  are known although it can be difficult to determine such values with sufficient accuracy. On the other hand it is possible to perform the bolus injection in a manner which produces an arterial concentration-time curve approximating a simple cosine function which after insertion into equation (8) leads to a manageable expression. Three experimentally useful functions, i.e.  $C_M^A(t) = kt$  (ramp infusion),  $C_M^A(t) = k \sin(\omega t)$  (square wave) and  $C_M^A(t) = C_M^A(t') \exp[-\lambda(t-t')]$  (bolus venous bolus injection) are listed in Table 1 with corresponding expressions of  $E(T)$  obtained according to equation (8). The square wave and ramp bolus injections give rise to expressions of  $E(T)$  with the blood flow of the brain as the only variable.

Ramp and square wave infusions were rejected in the present study, however, because the simultaneous investigation of blood-brain glucose transfer required that the concentration of labeled glucose in the cerebral vessels the time of termination of the experiment be known. Therefore *i.v.* bolus injection was used, and the approximation was made (for experimental confirmation see later) that the major part of the  $C_M^A(t)$  versus time curve butanol closely approximated a monoexponential decay function when butanol was administered as an *i.v.* bolus less than a second. Thus, if the  $C_M^A(t)$  vs. time curve is

Table 1. Arterial extraction fraction of freely diffusible indicator in brain as function of arterial termination

Function	$E(t)$
$C_b^*(t)$	$\exp\left[-\frac{F^m T}{\lambda_w}\right] \left\{ C_b^*(t) - \int_0^t C_b^*(t) \exp\left[-\frac{F^m(t-t')}{\lambda_w}\right] dt' \right\}$
$k$	$\frac{1 - \exp\left[-\frac{F^m T}{\lambda_w}\right]}{\left[\frac{F^m T}{\lambda_w}\right]}$
$\lambda$	$\left[\frac{F^m T}{\lambda_w}\right] \left\{ 1 - \frac{1 - \exp\left[-\frac{F^m T}{\lambda_w}\right]}{\left[\frac{F^m T}{\lambda_w}\right]} \right\}$
$C_b^*(t) \exp[-k(t-r)]$ $t > r$	$\left[\frac{k}{\left[\frac{F^m T}{\lambda_w} - k\right]} \left\{ \exp[-k(T-r)] - \exp\left[-\frac{F^m(T-r)}{\lambda_w}\right] \right\} \right]$

is exactly 1, i.e. to time  $T$ ) a monoexponential decay curve from time  $t = 0$ ,  $C_b^*(t) = C_b^*(0) \exp[-k(t-r)]$  for  $t > r$ , as shown in Table 1 for  $k \neq p$ .

$$C_b^*(t) = \frac{\exp[-k(T-r)] - \exp[-p(T-r)]}{(p-k) - \exp[-k(T-r)]} \quad (9)$$

which  $p$  equals  $F^m/\lambda_w$ . However, the  $C_b^*(t)$  curve gives the peak time  $r$ . For  $E(t)$  calculated from equation (9) is an acceptable approximation of the actual  $E(t)$  for period 0 to  $T$  it must therefore be shown that the error of labeled bolus in arterial blood prior to  $t$  is not cause  $E(t)$  calculated by means of equation (9) to greatly in error. It will be shown in Results that this is the case for  $T = 20$  s. By insertion of equation (9) in equation (7) it is possible to express the brain content of glucose as function of  $p$ ,  $k$ , and  $T$ .

$$\frac{\exp[-k(T-r)] - \exp[-p(T-r)]}{(p-k) - \exp[-k(T-r)]} = \frac{F^m C_b(T)}{\lambda_w Q_b(T)} \quad (10)$$

from which equation  $F^m$  can be calculated when  $C_b^*(t)$ ,  $\lambda_w$ ,  $Q_b(T)$ ,  $k$ ,  $T$  and  $r$  are known, provided the assumption underlying the equations (4) and (8) are reasonably satisfied.

In the present study the equations were used as fol-

low. Initially  $k$  and  $r$  were estimated from values of  $C_b^*(t)$ . Second  $p$  was estimated by fitting equation (9) to the experimentally determined values of  $E(t)$ . Third  $F^m$  determined from equations (1) and (5) was compared to  $F^m$  calculated from equation (10), using value of  $\lambda_w$  obtained directly by equilibration of blood and brain tissue with labeled bolus.

Two problems interfere with the calculation of  $p$  (and hence  $F^m$ ) from equation (10). First the method as presented does not yield  $k$  and  $r$  and second, equation (10) is transcendental equation which can be satisfied by one to no possible values of  $p$ . The problems were addressed as follows.

In routine application of the method the constant  $k$  and  $r$  are estimated by the simple expedient of measuring the arterial indicator concentration,  $C_b^*(T)$ , at the time of termination of the experiment.

$$C_b^*(t) = C_b^*(t) \exp[-k(t-r)], \quad t > r$$

then

$$C_b^*(T) = C_b^*(t) \exp[-k(T-r)],$$

and by insertion of the expression for  $C_b^*(t)$  derived from these two equations into equation (6) with  $C_b(T)$  replaced by  $Q_b(T)$ ,  $F^m$  by  $F_b^m$  and  $E_b(T)$  by 1



Following introduction of an indicator into the circulation the indicator is taken up per unit weight of any organ including brain at a rate equal to the product of the blood flow rate per unit organ weight  $F^M$  and the arteriovenous deficit

$$\frac{dC_M(t)}{dt} = F^M (C^M(t) - \bar{C}^M(t)) \quad (2)$$

where  $\bar{C}^M(t)$  is the organ venous blood concentration of the indicator at the time  $t$  after introduction of the indicator into the circulation. When brain uptake of the indicator is entirely flow-limited, i.e. equilibrium between tissue and venous concentrations occurs "instantaneously" the rate of change of brain indicator content can be expressed by the following modification (Kety 1951) of equation (2)

$$\frac{dC_M(t)}{dt} = F^M \left( C^M(t) - \frac{C_M(t)}{\lambda_M} \right) \quad (3)$$

in which  $\lambda_M$  is the tissue blood partition coefficient of the indicator (ml/g). Provided  $F^M$  is constant and the tissue is homogeneous with respect to  $F^M$  and  $\lambda_M$ , equation (3) can be integrated to yield

$$C_M(T) = F^M \exp \left[ F^M \frac{T}{\lambda_M} \right] \int_0^T C^M(t) \exp \left[ -F^M \frac{t}{\lambda_M} \right] dt \quad (4)$$

Equation (4) was used by Landau et al. (1955) to measure regional cerebral blood flow in the cat 60 s after an i.v. bolus injection of antipyrine. In this case tissue concentrations were measured autoradiographically, as was  $\lambda_M$ . In regions of brain sufficiently small to allow the authors to argue that the conditions of integration were fulfilled. In the present study  $\lambda_M$  and  $F^M$  will be measured in regions of brain approximately corresponding to major subdivisions of the rat brain (cerebellar hemisphere, vermis, pons, midbrain, basal ganglia, parietal cortex and frontal, occipital and temporal lobes). These regions consist chiefly of grey matter for which it has been shown that oxidative metabolism and by inference  $F^M$  does not vary much within each region (Sokoloff et al. 1977).

$E(T)$ , the net extraction fraction of indicator in the zero to  $T$  time interval, is defined by the following equation

$$E(T) = 1 - \frac{\int_0^T \bar{C}^M(t) dt}{\int_0^T C^M(t) dt} \quad (5)$$

Insertion of the numerator from equation (5) in equation (2) after integration readily yields the fundamental equation (1). Equation (1) applies to any organ as well as to the whole body provided  $F^M$  is constant. Thus for a unit weight of another organ indicated by subscript  $o$

$$C_o(T) = F^M E_o(T) \int_0^T C^M(t) dt \quad (6)$$

If  $C^M(t)$  for the two organs are identical for all values of  $t$  from 0 to  $T$

$$F^M = F^o \frac{E_o(T)}{E(T)} \frac{C_o(T)}{C_M(T)}$$

In the present method a reference organ is introduced in the form of a syringe into which blood is drawn at the rate  $F^M$ . In this case  $C_o(T)$  is substituted by  $Q(T)$ , the total radioactivity accumulated in the syringe at the time 0 to  $T$  and  $E_o(T)$  equals 1. Thus for the relationship between an organ and the syringe we have

$$F^M = F^M \frac{C_M(T)}{E(T) Q(T)}$$

Equation (7) is the operational form of the final version of the method. In whole-brain studies,  $E(T)$  can be determined directly by equation (5) following analysis of arterial and mixed venous blood. For regional experiments in which  $C^M$  for each region is unknown,  $E(T)$  can be determined by the following combination of equations (1) and (4)

$$E(T) = \frac{\exp \left[ -F^M \frac{T}{\lambda_M} \right] \int_0^T C^M(t) \exp \left[ F^M \frac{t}{\lambda_M} \right] dt}{\int_0^T C^M(t) dt}$$

which rests on the assumptions that underlie equation (1), i.e. homogeneity of  $F^M$  and  $\lambda_M$  in the region examined, equilibration of the flow indicator between tissue and venous blood and constancy of blood flow during the experiment.

Equation (8) can be solved numerically if sufficient numerous values of  $C^M(t)$  are known although it can be difficult to determine such values with sufficient accuracy. On the other hand it is possible to perform the integration in a manner which produces an arterial concentration-time curve approximating a simple cosine function which after insertion into equation (8) leads to a manageable expression. Three experimentally useful functions, i.e.  $C^M(t) = kt$  (ramp infusion),  $C^M(t) = k \exp(-kt)$  (square wave) and  $C^M(t) = C \exp[-k(t-1)]$  (bolus venous bolus injection) are listed in Table 1 with corresponding expressions of  $E(T)$  obtained according to equation (8). The square wave and ramp infusion functions give rise to expressions of  $E(T)$  with the blood flow of the brain as the only variable.

Ramp and square wave infusions were rejected in the present study however because the simultaneous equilibration of blood-brain glucose transfer required that the concentration of labeled glucose in the cerebral venous blood at the time of termination of the experiment be known. Therefore i.v. bolus injection was used and the approximation was made (for experimental confirmation, see later) that the major part of the  $C^M(t)$  versus time curve for butanol closely approximated a monoexponential decay function when butanol was administered as an i.v. bolus less than a second. Thus if the  $C^M(t)$  vs. time curve

the cerebral extraction fraction of freely diffusible indicator in brain as a function of arterial concentration

Function	Function	$E(T)$
$C_b^m(t)$	$C_b^m(t)$	$\exp\left[-\frac{f^m T}{\lambda_{br}}\right] \int_0^T C_b^m(t) \exp\left[\frac{f^m t}{\lambda_{br}}\right] dt$ $\int_0^T C_b^m(t) dt$
$k$	$k$	$\frac{1 - \exp\left[-\frac{f^m T}{\lambda_{br}}\right]}{\left[\frac{f^m T}{\lambda_{br}}\right]}$
$k$	$k$	$\frac{2}{\left[\frac{f^m T}{\lambda_{br}}\right]} \left[ 1 - \frac{1 - \exp\left[-\frac{f^m T}{\lambda_{br}}\right]}{\left[\frac{f^m T}{\lambda_{br}}\right]} \right]$
$C_b^m(t') \exp[-k(t-t')]$	$C_b^m(t') \exp[-k(t-t')]$	$\frac{k}{\left[\frac{f^m T}{\lambda_{br}} - k\right]} \left[ \exp[-k(T-t')] \exp\left[\frac{f^m (T-t')}{\lambda_{br}}\right] - \exp[-k(T-1)] \right]$

in artery to  $t$ , to time  $T$  monoexponential decay from time  $t'$  i.e.  $C_b^m(t) = C_b^m(t') \exp[-k(t-t')]$  for then, as shown in Table 1 for  $k \neq p$ .

$$\frac{\exp[-k(T-t')] - \exp[-p(T-t')]}{(p-k) \{1 - \exp[-k(T-t')]\}} \quad (9)$$

which  $p$  equals  $f^m/\lambda_{br}$ . However, the  $C_b^m(t)$  curve rises to a peak time  $t'$ . For  $E(T)$  calculated from equation (9) as an acceptable approximation of the actual  $E(T)$  for a period 0 to  $T$  it must therefore be shown that the locus of labeled bolus in arterial blood prior to  $t'$  is not cause  $E(T)$  calculated by means of equation (9) to greatly in error. It will be shown in Results that this is the case for  $T = 20$  s. By insertion of equation (9) in equation (7) it is possible to express the brain content of tracer as a function of  $p$ ,  $k$  and  $T$ .

$$\frac{\exp[-k(T-t')] - \exp[-p(T-t')]}{(p-k) \{1 - \exp[-k(T-t')]\}} \frac{f^m C_{br}(T)}{\lambda_{br} Q_a(T)} \quad (10)$$

in which equation  $f^m$  can be calculated when  $f^m_{br}(T)$ ,  $\lambda_{br}$ ,  $Q_a(T)$ ,  $k$ ,  $T$  and  $t'$  are known, provided the assumptions underlying the equations (4) and (8) are reasonably fulfilled.

In the present study the equations were used as follows.

Initially  $k$  and  $t'$  were estimated from values of  $C_b^m(t)$ . Second  $p$  was estimated by fitting equation (9) to the experimentally determined values of  $E(T)$ . Third,  $f^m$  determined from equations (1) and (5) was compared to  $f^m$  calculated from equation (10), using a value of  $\lambda_{br}$  obtained directly by equilibration of blood and brain tissue with labeled butanol.

Two problems interfere with the calculation of  $\lambda_{br}$  (and hence  $f^m$ ) from equation (10): First the method as presented does not yield  $k$  and  $t'$  and second, equation (10) is a transcendental equation which can be satisfied by one, two or no positive values of  $p$ . The problems were addressed as follows.

In routine application of the method the constants  $k$  and  $t'$  are estimated by the simple expedient of measuring the arterial indicator concentration,  $C_a^m(T)$  at the time of termination of the experiment.

$$C_b^m(t) = C_b^m(t') \exp[-k(t-t')], t > t'$$

then,

$$C_b^m(T) = C_b^m(t') \exp[-k(T-t')],$$

and by insertion of the expression for  $C_b^m(t)$  derived from these two equations into equation (6) with  $C_a(T)$  replaced by  $Q_a(T)$ ,  $f^m$  by  $F^m_{br}$  and  $E_a(T)$  by 1

Table 2. Comparison of solutions to equations (1) and (5) and equation (10) for  $T=20$  s,  $k_1=0.32$ ,  $\lambda_{av}=0.77$  ml  $g^{-1}$

$F^{MC}(T)$ $Q_d(T)$	$F^{MC}$ equations (1) & (5)	$F^{MC}$ equation (10) $k=0.04$ s	$k=0.07$ s	$k=0.1$ s
$102 \pm 9$	$133 \pm 1$	$123 \pm 13$	$135 \pm 16$	$147 \pm 70$

All other values are expressed in ml (100 g)  $\min^{-1} \pm$  S.E. ( $n=10$ ).

$$\frac{Q(T)}{F^{MC}} = \int_0^T C_m^*(t) \exp[k(T-t)] \exp[-k(T-t)] dt$$

or

$$\frac{Q(T)}{F^{MC}} = C_m^*(T) \frac{(\exp[k(T-t)] - 1)}{k} \quad (11)$$

in which the symbols represent the variables defined above. From equation (11)  $k$  can be calculated when the variables  $Q_d(T)$ ,  $F^{MC}$  and  $C_m^*(T)$  as well as  $k_1$  are known. The value of  $k_1$  was considered a constant for the following reasons. Both  $k$  and  $t$  are functions of the cardiac output and a central volume of distribution located between the site of injection and the site of arterial blood collection. When the ratio between these two variables increases  $t$  becomes smaller and  $k$  greater (and the opposite changes occur when the ratio is reduced). The value of  $k_1$  probably approximates the ratio between  $t$  and the time of passage of blood between the injection and sampling sites.

Thus only two constants need to be known in advance of blood flow measurement:  $k_1$  and  $\lambda_{av}$ .

The second problem involves the number of solutions to equation (10). When  $C_m^*(t)$  decays approximately monoexponentially and  $T$  exceeds a certain value a given concentration of indicator in brain at time  $T$  may arise from a relatively low blood flow which carries a relatively small amount of indicator to brain but provides basis for retention, or from a relatively high blood flow which carries a relatively high amount of indicator to the brain but results in a lower degree of retention. However, the particular shape of the curve of  $C_m^*(T)$  as a function of  $p$ ,  $k$  and  $T$  (equation (10)) reveals that only one positive value of  $p$  satisfies equation (10) when

$$\frac{C_m^*(T)F^{MC}}{\lambda_{av}Q_d(T)} \leq \frac{k}{\exp[-k(T-t)] - 1}$$

Thus the possibility of two solutions depends on the magnitude of  $T$  and the rate of decay of  $C_m^*(t)$ . In Table 2 solutions to equation (10) for different values of  $k$  and  $T=20$  s, and  $k_1=0.32$ , are compared with values of  $F^{MC}$  calculated directly from equation (1), using  $E(T)$  values determined experimentally by collection of arterial and cerebral venous blood (cf. equation (5)). For the values of

$k$ ,  $T$  and  $k_1$  chosen (it is evident that only a single value satisfies equation (10))

#### Determination of blood-brain glucose transfer

Equations (1), (5) and (7) also apply to substances which penetrate into the extravascular compartment and only leave these by way of the venous blood. The equations also apply in the case of D-glucose which enters the brain by facilitated diffusion (Crosby 1966). Following  $i.v.$  bolus injection of tracer glucose a certain quantity of tracer is taken up by brain at the time  $T$ . If no tracer glucose has yet left the brain at that time (no back-diffusion) and if no tracer glucose remains in the capillaries,  $E(T)$  equals the unidirectionally extracted fraction of glucose in brain henceforth to be known as  $E_{un}$ . It is likely that  $E_{un}$  is constant for some time following injection and the large sink action of the extracellular and intracellular spaces for glucose (Borchardt 1970; Lund-Andersen & Hjeltnes 1977) cannot extract glucose to escape from brain in the short period of 20 s (Betz et al. 1973).

If  $C_p^*$  is the total concentration of glucose in arterial plasma, equation (7) yields

$$J_{un} = E_{un} \cdot F^{MC} C_p^* = C_m^* F^{MC} \frac{C_p^*(T)}{Q_d(T)}$$

where  $J_{un}$  represents the unidirectional blood-brain glucose flux per unit weight of brain,  $C_p^*(T)$  the extracellular content of labeled glucose per unit weight of brain,  $Q_d(T)$  the quantity of labeled glucose collected in arterial sampling syringe. Since glucose in plasma is bound to erythrocytes with glucose in erythrocytes (Heath & Rose 1969)  $F^{MC}$  and  $F_p^*$  refer to the respective plasma flow rates. The concentration  $C_p^*$  does not represent the concentration at which the transport occurs, different parts of the capillary.

It is important to emphasize that  $C_p^*(T)$  must be the concentration of glucose in the vascular compartment of the brain; small amounts of labeled glucose were present in the rest of the brain, these amounts were subtracted from contents of labeled glucose in brain in order to obtain  $C_p^*(T)$ . The amount of labeled glucose in the capillaries per unit weight of brain at the time  $T$  is estimated as  $V^M C_p^*(T)$  in which  $V^M$  is the plasma volume per unit weight of brain. Thus  $C_p^*(T)$  the amount of labeled glucose located extravascularly in brain at time  $T$  was calculated as

$$(T) = C_b(T) - V^m C_p^m(T) \quad (13)$$

which  $C_b(T)$  is the total amount of labeled glucose in 1 cgt of brain,  $V^m$  is equated with the mannitol volume alone at time  $T$  obtained in separate expt described below, mannitol was chosen because the brain permeability is very low (Lund-Andersen 1961).

## METHODS

Use of equation (10) for calculation of cerebral blood flow requires that the constants  $k_1'$  and  $\lambda_{ex}$  be known. To measure  $Q_{brain}$  the injected dose of indicator  $F$ , the rate of sampling by the arterial catheter  $C_b(T)$ , the concentration of indicator per unit weight of brain,  $Q_b(T)$ , amount of indicator in the arterial sampling syringe  $Q_s(T)$ , the concentration of indicator in arterial blood  $C_a(T)$ , the concentration of indicator in arterial blood time  $T$  and  $T$  the sampling and decapitation time are determined experimentally. Separate expts were carried out to determine  $k_1'$  and  $\lambda_{ex}$ .

### Determination of values of its components

Arterial concentration-time curves for butanol were obtained in 3 rats by drawing arterial blood continuously into a 100 µl catheter in such a manner as was aided by suction of individual samples by air bubbles. The samples were transferred to counting vials and prepared as described below. The initial dilution of arterial plasma used by the holes injection is estimated from the peak area of the arterial concentration time curves and the  $C_a$  concentration in the syringe.

The identity of concentration-time integrals obtained in the femoral and the left common carotid artery was assumed in four rats following cannulation of the femoral artery and the left external carotid artery. Ratios of time integrals in the three vessels were calculated.

The ECT of butanol was measured directly according to equation (5) in 10 rats, using microelectrodes of  $T$  ranging from 3 to 70 µm in order to avoid any interference of carotid cannulation with cerebral blood flow. Blood was drawn from the femoral artery and the superior sagittal sinus in the torcular in these expts.

### Determination of $k_1'$ , $\lambda_{ex}$ (butanol), and $V^m$

The variables  $k_1'$  and  $\lambda_{ex}$  were determined in three rats by using a monoexponential decay curve to the experimentally determined values of  $C_p^m(T)$  for butanol.  $\lambda_{ex}$  was measured directly following prolonged equilibration of butanol in brain tissue *in vivo*. The samples of brain tissue corresponded to the samples used for blood flow determination and gave an estimate of the variation of  $\lambda_{ex}$  between different regions. The equilibration of butanol between brain and blood was performed as follows.

In three rats, a mixture of 15 µl 17 µCi  $\mu\text{l}^{-1}$   $n$ -[1- $^{14}\text{C}$ ] butanol and 1500 µl unlabeled ethanol was slowly infused for 45 min, using fractionated syringe schedule designed to yield an approximately constant arterial concentration of butanol (Sokoloff et al 1971). The ethanol was included

to exhaust the alcohol dehydrogenase system capacity for breakdown of alcohol during the expt and frequent arterial blood samples were taken to ensure the constancy of the arterial concentration. At the end of the expt, the animal was decapitated, and samples of brain and arterial blood removed and assayed for radioactivity per unit weight of brain as described below. The partition coefficient was calculated as the unit weight of brain to unit volume of blood radioactivity ratio.

In 7 rats cerebral blood flow and cerebral content of labeled mannitol were determined simultaneously 70 s after a bolus injection of a mixture of labeled butanol and labeled mannitol as described below. The arterial plasma concentration of mannitol 20 s after the injection was determined by measuring the mannitol concentration in the last portion of blood collected through the femoral arterial catheter in the manner described below.  $V^m$ , the plasma volume per unit weight of brain, was calculated as the ratio between  $C_b(T)$  and  $C_p^m(T)$  for labeled mannitol.

In the above derivation, it was assumed that mannitol does not cross the blood-brain barrier. If mannitol does diffuse into the extravascular space to some extent, the calculated plasma volume per unit weight of brain would include small apparent extravascular volume of distribution of mannitol. If labeled D-glucose diffuses passively across the blood-brain barrier to the same extent, application of the mannitol distribution volume for  $V^m$  in calculation of  $C_b(T)$  for labeled glucose will eliminate errors from passive blood-to-brain diffusion of D-glucose.

### Determination of regional cerebral blood flow and blood-brain glucose transfer

In 3 rats, set of  $F$ ,  $F_1$ ,  $C_b(T)$ ,  $C_a(T)$ ,  $Q_b(T)$ ,  $Q_s(T)$ ,  $C_a(T)$ ,  $Q_{man}$ ,  $C_p^m(T)$ , and  $T$  were measured.  $C_b(T)$  was calculated by means of equation (14). Then, regional and whole-brain blood-flow and regional and whole-brain blood-brain glucose transfer rates were calculated by iteration from equation (10) and (12) using values of  $k_1'$  and  $\lambda_{ex}$  obtained as mentioned above and calculating  $k$  by equation (11).

### General procedure

The 350 g male Wistar rats were kept specific-pathogen-free with unlimited access to food and water. Anaesthesia was induced with ether and the animals were paralyzed with 3 mg/kg saxanethonium, intubated and ventilated with 1% halothane vaporized in a mixture of nitrogen and oxygen (70:30).

Catheters were placed in the tail artery, both femoral arteries, and one femoral vein. As described above the superior sagittal sinus was cannulated in some rats. Cannulation of the superior sagittal sinus was performed in the following manner. A small burr hole was placed immediately above the torcular care being taken not to rupture the dura. By means of microaspirator capillary tube drawn out to fine tip was passed so gently through the dura that no leakage around the tube was observed. Arterial blood was assayed for pH,  $P_{aO_2}$  and  $P_{aCO_2}$  with microelectrodes (Radiometer Copenhagen).

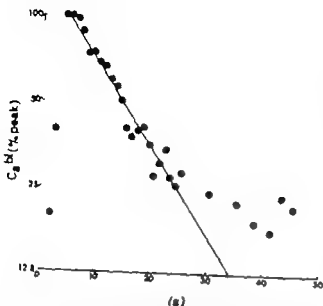


Fig. 1 Typical arterial concentration curve (logarithmic ordinate scale) for butanol following an i.v. bolus injection of 700  $\mu$ l. Due to method of sampling peak activity was always represented in one of samples. Points regressed to equation  $C_B(t) = C_B(4) \exp(-0.07(t-4))$  the correlation coefficient  $r=0.98$  in which  $t$  is expressed in s. Concentrations are relative to peak concentration.

The animals were considered to be in respiratory steady state when  $P_{aCO_2}$  in samples taken at least 10 min apart agreed within 10%. Arterial blood pressure was continuously recorded with a strain gauge transducer.

When the animals were in respiratory steady state with  $P_{aO_2}$  30–35 mmHg,  $P_{aCO_2}$  > 100 mmHg and mean arterial blood pressure > 100 mmHg, a mixture of 5  $\mu$ Ci [ $^3$ H]butanol and 70  $\mu$ Ci [ $^3$ H]glucose in 200  $\mu$ l Ringer HEPES buffer solution (Gjefde & Crooe 1975) was injected into the femoral vein in less than a second. Prior to mixing the [ $^3$ H]glucose solution was air-dried and resuspended to eliminate volatile  $^3$ H-activity.

Integration of concentration-time curves in blood was produced mechanically as described previously (Gjefde et al. 1975). Briefly the samples were drawn into calibrated glass syringes at a known and constant rate during and for 20 s after i.v. injection. At the end of 20 s withdrawal was discontinued and the animal decapitated with a rodent guillotine. The brain was rapidly removed and dissected bilaterally into the following regions: weighing approximately 75 mg each, cerebellar hemisphere, cerebellar vermis, medulla and pons, midbrain, basal ganglia, parietal cortex, and frontal, occipital and temporal lobes.

The blood and tissue samples were rapidly transferred to pre-weighed counting vials containing 1.5 ml of a Solvène (Packard) and isopropanol mixture (1:1). The vials and samples were then reweighed, blanked with 0.5 ml 3% hydrogen peroxide and stored overnight at 50°C. The next morning preparation was completed by addition of 20 ml of an Instagel (Packard) and 0.5 N HCl mixture (1:1). The vials were allowed to stabilize at counting temperature for 24 h prior to counting in a Tri-Carb 4-5 (Packard) liquid scintillation spectrometer (Hendler 1964).

Plasma concentrations of glucose were measured freshly sampled arterial plasma by the glucose oxidase method (Christensen 1967; Hjelm & De Vries 1971). Arterial hematocrit, used for calculation of plasma concentrations of labeled glucose and insulin from blood concentrations of labeled glucose and insulin, was determined by centrifugation.

Preparations of  $n$ -[1- $^{14}$ C]butanol, D-[1- $^3$ H]glucose and D-[1- $^3$ H]glucose were obtained from New England Nuclear Corporation with specific activities of 100 mmol  $^{-1}$  18 Ci mmol $^{-1}$  and 1–10 Ci mmol $^{-1}$  respectively.

## RESULTS

### Determination of constants and confirmation of assumptions

A representative arterial concentration-time curve for butanol is shown in Fig. 1. The three curves obtained in this manner confirmed that the decay of butanol was approximately monoexponential from 4 to 20 s after the injection as required by equation (9). The variables  $k$  and  $t$  averaged  $0.069 \pm 0.006$  (S.E.  $n=3$ ) and  $4.7 \pm 0.7$  s (S.E.  $n=3$ ), respectively. Multiplication of  $k$  and  $t$  yielded a value of  $0.33 \pm 0.06$  (S.E.).

Integration of the curves revealed that the integral prior to peak time represented 18–19% of the total integral from 0 to 70 s. The combined error made in the calculation of  $E(T)$  by assuming  $C_B$  to be negligible for  $t < t_p$  as well as monoexponentially decaying for  $t_p \leq t \leq T$  was estimated as follows. With the three arterial curves determined above  $E(T)$  was estimated numerically according to equation (8) using an average value for  $(F^0/\lambda_{av})$  of 0.028 s. The values of  $E(T)$  calculated in this manner were compared with values of  $E(T)$  calculated according to equation (9) using the relevant value of  $k$  and the value of  $(F^0/\lambda_{av})$  used above. The ratio of the former values of  $E(T)$  to the latter values of  $E(T)$  represents the factor by which blood flow and

Table 3 Integrals of arterial concentration-time curves for butanol determined in right and left femoral arteries relative to integral determined in right common carotid artery

Artery	Ratio $\pm$ S.E. ( $n=4$ )
Right femoral	0.99 $\pm$ 0.03
Left femoral	1.0 $\pm$ 0.04

Curves were integrated for periods of 10–30 s after intravenous injection of a 700  $\mu$ l. bolus of labeled butanol.

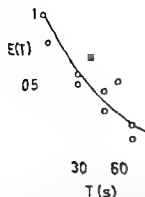


Fig. 2. Directly measured and extrapolations of labeled brain,  $E(T)$ , vs. the time of measurement  $T$  from data result of least-squares computerized operations of equation (9) to points shown. Curves correspond to  $p=0.139$ ,  $k=0.059$  and  $t'=4.6$  s.

underestimated when calculated according to the described approximation procedures. This factor averaged  $0.973 \pm 0.025$  (S.E.,  $n=3$ ).

From the concentration of radioactivity in the plasma, the minimal dilution of the bolus was estimated to be 50-fold. Hence the glucose concentration in plasma was not significantly reduced by the bolus injection.

The amount of butanol measured in samples taken from each femoral artery relative to that measured in blood sampled from the right external carotid artery is recorded in Table 3. The ratios confirmed that the arterial concentration-time curve integrals were equal in the three arteries for periods ranging from 10 to 30 s after injection of the bolus.

$E(T)$ -values of butanol directly measured for various values of  $T$  in two rats are shown in Fig. 2. With  $p=0.139$  s $^{-1}$  a least-squares computerized operation (Gjedde & Crone 1975) of equation (9) to the experimental values of  $E(T)$  yielded a value of  $p$  of  $0.139$  s $^{-1}$ . For  $k_{app}=0.77$  ml g $^{-1}$  min $^{-1}$  this value corresponded to  $k=139$  ml (100 g) $^{-1}$  min. Using the  $E(T)$ -values and the corresponding measured  $C_p(T)$ -values for calculation of  $F_{app}$  from equation (1), the result came to  $133 \pm 12$  ml (100 g) $^{-1}$  min (S.E.). As shown in Table 2, variation of  $k$  from 0.14 to 0.1 s $^{-1}$  did not significantly affect the close agreement.

The average brain-blood partition coefficients for regions of brain are shown in Table 4. They did not significantly differ from the whole-brain average of  $0.770 \pm 0.072$  ml g $^{-1}$  (S.E.M.), indicating homogeneity of brain with respect to  $k_{app}$ .

Table 4 Brain-blood partition coefficients for butanol

Region	$k_{app}$ (ml g $^{-1}$ )
Cerebellar hemisphere	$0.774 \pm 0.020$
Cerebellar cortex	$0.774 \pm 0.032$
Medulla and pons	$0.777 \pm 0.032$
Midbrain	$0.744 \pm 0.026$
Basal ganglia	$0.746 \pm 0.033$
Parietal cortex	$0.790 \pm 0.040$
Occipital lobe	$0.819 \pm 0.063$
Temporal lobe	$0.775 \pm 0.037$
Frontal lobe	$0.791 \pm 0.038$

$\pm$ S.E. (3).

The plasma volume (mannitol distribution volume) per unit weight of whole-brain averaged  $1.07 \pm 0.12$  ml (100 g) $^{-1}$  ( $\pm$ S.E.) at a plasma flow of  $46 \pm 9$  ml (100 g) $^{-1}$  min corresponding to a mean transit time of 1.14 s. At the labeled glucose concentration in plasma at time  $T$  measured below these values corresponded to the presence in plasma of  $8.6 \pm 0.4\%$  of all labeled glucose in the brain.

#### Determination of regional cerebral blood flow and blood-brain glucose transfer

The mean arterial blood pressure, blood gases, pH, hematocrit, arterial plasma glucose concentration and cardiac output of five rats are shown in Table 5.

Table 5 Blood pressure, blood gases, pH, hematocrit, arterial plasma glucose and cardiac output of rats used in the present study

Variable	Mean $\pm$ S.E. ( $n=5$ )
MABP (mmHg) $^a$	$100 \pm 3$
$P_{aO_2}$ (mmHg) $^b$	$130 \pm 15$
$P_{aCO_2}$ (mmHg) $^c$	$33 \pm 2$
pH $^d$	$7.47 \pm 0.02$
hct $^e$	$0.44 \pm 0.02$
$C_p^f$ (mmol l $^{-1}$ ) $^g$	$10.4 \pm 0.9$
$F_{app}$ (ml kg $^{-1}$ min $^{-1}$ ) $^h$	$139 \pm 5$

Mean arterial blood pressure. Arterial oxygen tension. Arterial carbon dioxide tension. Arterial pH. Hematocrit. Arterial plasma glucose concentration. Cardiac output was determined by extrapolation of the monoexponential arterial decay curve beyond the time of onset of recirculation. Thus if  $F_{app}$  is the cardiac output and  $Q_{app}$  the total administered dose of labeled butanol, for  $p=0.139$  s $^{-1}$ ,  $Q_{app} = F_{app} \int_0^\infty C_p(t) \exp(-kt) dt$ . Since  $Q_{app} = F_{app} \int_0^\infty C_p(t) \exp(-kt) dt$ , then, by division,  $F_{app} = \int_0^\infty C_p(t) \exp(-kt) dt / \int_0^\infty C_p(t) \exp(-kt) dt$ , from which equation cardiac output was calculated when  $k$  was known.

Table 6 Whole-brain blood flow, blood-brain glucose flux and tracer glucose extraction

Variable	Mean $\pm$ S.E. (n=5)
$F^M$ (ml (100 g) <sup>-1</sup> min <sup>-1</sup> )	179 $\pm$ 7
$J_{glc}$ ( $\mu$ mol (100 g) <sup>-1</sup> min <sup>-1</sup> )	144 $\pm$ 13
$E_{glc}$	0.1 $\pm$ 0.01

Whole brain blood flow \* Blood-brain glucose flux  
Tracer glucose extraction

In these rats the following values for butanol and labeled glucose were determined at  $T=20$  s ( $\pm$  S.E.):  $F^M=0.0917 \pm 0.0016$  ml min<sup>-1</sup>  $Q_{glc}(T)=2.60 \pm 0.11$  nCi  $C_{glc}^b(T)=2.94 \pm 0.22$   $\mu$ Ci (100 g)<sup>-1</sup>  $Q(T)=18.7 \pm 0.7$  nCi  $C_{glc}^b(T)=6.07 \pm 0.45$   $\mu$ Ci (100 g)<sup>-1</sup>  $C_{glc}^b(T)=74 \pm 6$  nCi ml<sup>-1</sup>  $C_{glc}^{tr}(T)=500 \pm 67$  nCi ml<sup>-1</sup> and  $Q_{total}=7.1 \pm 0.5$   $\mu$ Ci kg<sup>-1</sup>

Using  $kt=0.32$  the variable  $k$  was calculated from equation (11) to be  $0.058 \pm 0.008$  s<sup>-1</sup>

With  $kt=0.37$   $\lambda_{glc}=0.77$  ml g<sup>-1</sup> and  $k=0.058$  s<sup>-1</sup> equation (10) was used to estimate cerebral blood flow by iteration. Blood-brain glucose flux was calculated by means of equation (12)

Whole-brain blood flow and unidirectional blood-brain glucose flux are recorded in Table 6

The distribution of blood flow and blood-brain glucose transfer rates per 100 g of tissue in nine regions relative to the whole-brain average are shown in Fig. 4. Gross regional perfusion coefficients were highest in the midbrain and lowest in the cerebellar hemispheres and frontal lobe. Blood-brain glucose flux was highest in the parietal cortex, the only purely cortical region sampled.

## DISCUSSION

The present study shows that it is possible to measure regional cerebral blood flow and blood-brain glucose transfer simultaneously by a rapid experimental procedure.

The whole-brain blood flow rate measured here agrees with values obtained by Sakurada et al. (1978) and is approximately 30% higher than the value reported by Gjedde et al. (1975).

The unidirectional blood-brain glucose transfer observed in this study is twice as high as values for the rat published by Bachelard et al. (1973) and Pardridge and Oldendorf (1977).

## Cerebral blood flow

The present blood flow method resembles the microsphere technique but has the advantage that the indicator can be injected i.v. i.e. without any circulatory perturbation (Gjedde et al. 1977). The microcirculation of the brain is not affected.

The assumption that brain uptake of butanol is entirely flow-limited at the flow rates encountered in the present study was confirmed by the observation of good agreement between the value of  $k_{ex}$  estimated from data for a single bolus passage of butanol and the value determined directly from equilibration between blood and brain tissue. The butanol is suitable for measurements of  $F^M$  at perfusion rates about 100 ml (100 g)<sup>-1</sup> min<sup>-1</sup>. Raab et al. (1976) arrived at the same conclusion at somewhat lower cerebral blood flow rates in the monkey.

The whole brain blood flow measured here applies to rats in halothane anesthesia, a condition known to increase cerebral blood flow (Gjedde & Hindfelt 1975).

Van Ulbert & Levy (1978) used the same method to measure regional cerebral blood flow in the  $CO_2$ -response in the awake gerbil. They delayed 10 s after the  $CO_2$  injection  $E(T)$  was found to be at least 95% in normal gerbils and was consequently considered to be unity. Since  $E(T)$  decreases with increasing flow, it is likely that the  $CO_2$ -response was underestimated.

The regional distribution of blood flow rate showed only moderate variations. The highest

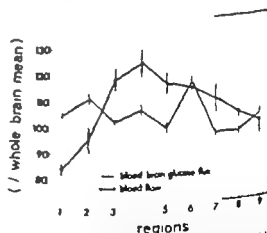


Fig. 3 Gross regional brain blood flow and blood-brain glucose transfer rates in rat, expressed relative to whole-brain means given in Table 5. Regions represent: 1 cerebellar hemisphere, 2 cerebellar vermis, 3 medulla and pons, 4 midbrain, 5 basal ganglia, 6 parietal cortex, 7 occipital lobe, 8 temporal lobe, 9 frontal lobe. Bars represent S.E.

tral blood flow was observed in the midbrain, according to results published by Sakurada et al. (1973).

# and brain glucose transfer

The present method resembles the method used by Bachelard et al. (1973) but is a single injection rather than a constant infusion experiment. The present method eliminated several disadvantages. In the study of Bachelard et al., the arterial glucose concentration was kept constant for 3 minutes during each period unknown amounts of radioactivity escaped from brain.

Single carotid injections performed rapidly as required by the Oldendorf technique (Pardridge & Oldendorf 1977), cause unknown increases of arterial blood pressure that may exceed the autoregulatory limit of autoregulation and cause temporary increases of cerebral blood flow.

Previous studies for glucose transport were measured in rats anesthetized with barbiturate or other agents known to reduce cerebral blood flow and thereby also the unidirectional glucose flux by a reduction of the capillary surface area available for blood-brain glucose transport.

The rate of glucose transport into brain observed in the present study agrees with the rate of uptake of 2-deoxy-D-glucose in brain of awake rat, reported by Sokoloff et al. (1977).

In conclusion, it is possible to measure cerebral blood flow and blood-brain glucose transfer rates by short-lasting procedure. The short time is important to studies of the regulation of cerebral metabolism. The rates of blood flow and glucose supply are essential to the metabolism of the brain because increases of cerebral metabolic rate depend on adequate delivery of metabolic substrates. Thus in studies of cerebral metabolism measurements of blood flow and glucose supply must be short enough to detect rapidly changing functional states.

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# Rapid steady state analysis of blood-brain glucose transfer in rat

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GJEDDE, A. Rapid steady-state analysis of blood-brain glucose transfer in rat. *Acta Physiol Scand* 1980, 108, 331-339. Received 17 Jan. 1979. ISSN 0001-6772. 1 Institute of Medical Physiology, Dept. A, University of Copenhagen, Denmark.

A new kinetic analysis of blood-brain glucose transport is described based on a steady-state model that takes account of cerebral blood flow, mean capillary glucose concentration, and cerebral metabolic rate. The maximal rate ( $T_{max}$ ) and half-saturation constant ( $K_m$ ) of glucose transport from blood to brain were determined in rats by measuring the rate of blood-to-brain glucose transfer at different blood glucose concentrations. Each determination lasted 20 seconds. For whole-brain,  $T_{max}$  and  $K_m$  averaged  $258 \pm 33$  (S.E.)  $\mu\text{mol} (100 \text{ g})^{-1} \text{ min}^{-1}$  and  $3.9 \pm 1.6$  (S.E.)  $\text{mmol l}^{-1}$  respectively. The regional variations were insignificant. The new approach permits kinetic parameters to be measured locally in brain in rapidly changing functional states.

**Key words:** D-glucose, blood-brain barrier, facilitated diffusion, hyperglycemia, hypoglycemia.

Glucose, the major nutrient of brain, passes from blood to brain by facilitated diffusion (Crone 1960). Limited blood-brain glucose transport has attracted attention as a possible contribution to cerebral metabolic dysfunction in hepatic encephalopathy (Lasser et al. 1975), Reye's syndrome (Haymond et al. 1976), and salicylism (Thurston et al. 1970). Kinetic analysis of blood-brain glucose transfer requires knowledge of blood flow, plasma glucose concentration profile in cerebral capillaries, and mean extracellular glucose concentration. Optimally, these variables should be determined simultaneously but previous kinetic analyses in rat have suffered from incomplete knowledge of one or more of the variables (Bachelard et al. 1973; Brender et al. 1975; Bianchi et al. 1970; Oldendorf 1971). Either blood flow was left undetermined or average capillary glucose concentration was equated with the arterial glucose concentration. The glucose concentration in brain extracellular water is low (A. Gjedde, A. J. Hansen & I. A. Silver unpublished) but the effect of different concentrations is relatively unknown.

A tracer method has been developed which allows the simultaneous determination of cerebral blood flow (whole-brain and regional) and unidirectional blood-brain glucose transfer by a procedure

lasting 20 s. Normal values for rat were presented previously (Gjedde et al. 1980).

In the present study the steady-state glucose concentration in arterial blood was varied by administration of insulin or glucose and the rates of blood-brain glucose transfer at different concentrations were used to calculate the kinetic constants for the facilitated diffusion of glucose from blood to brain. Calculation of the kinetic constants was based on mathematical treatment of the conventional single-capillary model of the cerebral circulation.

## METHODS

Briefly, bolus injections of a mixture of labeled bolus and tracer glucose were given in the femoral vein of rats. Arterial blood was slowly withdrawn into a syringe for 20 s. At the end of the collection the rats were decapitated and the brain rapidly removed for further processing.

### Theory

Uptake of radiolabeled glucose by brain following an intravenous bolus injection may be treated mathematically in the manner described by Bradbury et al. (1975) for tracers that enter brain by simple diffusion, and by Bass et al. (1976) for the enzymatic elimination of galactose in liver. Both approaches treat single capillary situation.

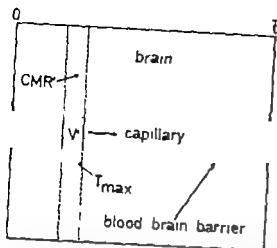


Fig. 1 Schematic representation of single-capillary model of brain used to treat blood brain glucose transport mathematically. The volume  $V$  enters at time 0 and exits at time  $t$ . Assumptions about the model are explained in the text.

Assume that backflux of labeled glucose from brain to capillary is negligible within 20 s, that axial diffusion within the capillary is negligible, that there is complete cross-stream mixing within the capillary, i.e. Taylor effects (Lassen & Croce 1970) are not too important, that a single "average" capillary with surrounding coaxial cylindrical tissue mantle can represent the brain as a whole, that the animal is in a steady-state with respect to the concentration of glucose in arterial plasma and the plasma flow rate of the brain, and that erythrocytes do not exchange glucose with plasma. Further assume that glucose is transported from blood to brain through a functionally single barrier by means of a symmetrical carrier, that the glucose transport capacity and the tissue rate of glucose metabolism are uniformly distributed along the length of the capillary, and that the simple diffusion permeability of the blood brain barrier for glucose is negligible (Lund Andersen 1979).

Consider an "average" capillary of length  $L$  with surrounding tissue (Fig. 1). Let  $C_p^u$  and  $C_p^l$  represent the plasma concentrations of unlabeled and labeled glucose respectively in the arterial end of the capillary. Following a bolus injection of tracer into a distant vein, a minute fraction of the bolus will reach the capillary. Let  $V$  be so small a volume of plasma (containing a fraction of the bolus that passes through the capillary) that longitudinal concentration gradients within  $V$  are negligible. Throughout its passage  $V$  is in contact with a capillary surface with a glucose transport capacity  $T_{max}$  and supplies a volume of tissue with a rate of glucose metabolism  $CMR_{glc}$ . Accordingly

$$\frac{V}{V} \cdot \frac{T_{max}}{T_{max}} = \frac{CMR_{glc}}{CMR_{glc}}$$

where  $V$ ,  $T_{max}$  and  $CMR_{glc}$  represent capillary plasma volume, glucose transport capacity and glucose consumption per unit weight of whole brain or a specified part of brain. The linear velocity of  $V$  equals  $f^p/(nA)$  where

$f^p$  is the plasma flow and  $n$  the number of capillaries per unit weight of brain, and  $A$  the average plasma cross-sectional area of brain capillaries. The plasma travel time can be expressed as

$$t = nAL/f^p = V/f^p$$

By the definitions given above

$$T_{max}/V = T_{max}/V = n$$

and

$$CMR_{glc}/V = CMR_{glc}/V = b$$

Provided  $T_{max}$  and  $K_m$  are independent of  $C$ , the local concentration of glucose in brain extracellular fluid, the following equations relate the movement of labeled and unlabeled glucose from blood to brain during its passage of the bolus containing labeled and unlabeled glucose (Wulbrandt & Rosenberg 1961):

$$V \frac{dC^{*l}}{dt} = -T_{max} C^{*l} / (K_m + C^{*l})$$

and

$$V \frac{dC^u}{dt} = -T_{max} \left( \frac{C^u}{K_m + C^u} - \frac{C^l}{K_m + C^l} \right)$$

in which  $K_m$  is the half saturation constant of the stated diffusion process,  $C^{*l}$  the concentration of glucose in the volume  $V$ , and  $C^{*u}$  the concentration of labeled glucose in  $V$ , both functions of time spent in the capillary. The provision that  $T_{max}$  and  $K_m$  be constant, albeit curved below,  $T_{max}$  and  $K_m$  are operational constants borrowed from enzymology and used for the experimentally derived maximal rate and half-saturation concentration of the transendothelial transfer mechanism at steady state. The local consumption of glucose equals the difference between inward and outward fluxes of labeled glucose across the blood-brain barrier. Assuming  $CMR_{glc}$  to be the same at all points along the capillary

$$V \frac{dC^{*l}}{dt} = -CMR_{glc} \quad (5)$$

After substitution of  $CMR_{glc} = V$  in and integration from  $[0, C_p^u(0)]$  to  $[t, C^u(t)]$

$$C^u = C_p^u - bt \quad (6)$$

This result expresses the fact inherent in the assumption that unlabeled glucose falls linearly from the arterial to the venous end of the capillary in the steady-state.

By inversion of (4) in (1)

$$\frac{dC^{*l}}{C^{*l}} = \frac{-n dt}{K_m + C_p^u - bt} \quad (7)$$

and by integration of (7) from  $[0, C^{*l}(0)]$  to  $[t, C^{*l}(t)]$

$$\ln \left[ \frac{C^{*l}(t)}{C^{*l}(0)} \right] = -b \ln \left[ \frac{K_m + C_p^u - bt}{K_m + C_p^u} \right]$$

which  $C_p^*/C_p^{**}$  represents the transmitted fraction of labeled glucose. After substitution of  $L$ ,  $a$ , and  $b$ , and rearrangement,

$$E = 1 - \exp \left[ \frac{T_{\text{max}}}{\text{CMR}_{\text{glc}}} \ln \left( 1 + \frac{\text{CMR}_{\text{glc}}}{(K_{\text{m}} - L_p)} \right) \right] \quad (7)$$

which  $E_{\text{glc}}$  the extracted fraction of tracer glucose when as tracer glucose enters  $V$  from the brain may its passage through the capillary. Equation (7) describes estimation of the relationship between  $T_{\text{max}}$  and  $K_{\text{m}}$  if the sets of  $E_{\text{glc}}$ ,  $\text{CMR}_{\text{glc}}$ ,  $L_p$  and  $C_p^*$  are known. If  $\text{CMR}_{\text{glc}}/(P^*K_{\text{m}} - C_p^*)$  does not exceed 0.1 as was shown to be the case, equation (7) can be simplified into the  $\text{CMR}_{\text{glc}}$

$$E = 1 - \exp \left[ \frac{T_{\text{max}}}{(P^*K_{\text{m}} - C_p^*)} \right] \quad (8)$$

Equation (8) shows that the concentration of labeled glucose has an almost isoequimolar fall along the capillary, and then has a profile along the capillary differential in that of unlabeled glucose.

Corresponding values of  $T_{\text{max}}$  and  $K_{\text{m}}$  can be determined after variation of  $C_p^*$  or  $P^*$  provided  $T_{\text{max}}$  is constant. Equation (8) is also the equation used by Crone (1973) to determine simple diffusion permeabilities for solutes by the indicator diffusion method.  $E_{\text{glc}} = \exp(-PST^*)$  here  $PS$  corresponds to  $T_{\text{max}}/(C_p^* - C_p^{**})$ . The equation shows that the decrease in plasma glucose concentration as blood passes along the capillary has little influence on the magnitude of the calculated kinetic constants when the ratio  $\text{CMR}_{\text{glc}}/(P^*K_{\text{m}} + P^*C_p^*)$  is small. When equation (8) applies, a plot of  $E_{\text{glc}}$  is  $(1 - E_{\text{glc}})$  versus  $-C_p^*/(P^*K_{\text{m}} - C_p^*)$  fits a straight line with the slope  $-K_{\text{m}}$  and ordinate intersection  $T_{\text{max}}$ .

#### Measurement of cerebral blood flow and blood-brain glucose transfer

Arterial and regional cerebral plasma flow rates,  $P^*$  were calculated from tissue and arterial blood sample contents of radioactive butanol, using the equation,

$$P^* = \frac{1}{E(T)} F_p^* \frac{C_p(T)}{Q_p(T)} \quad (9)$$

in which  $E(T)$  the net cerebral extraction fraction of radioactive butanol at the time  $T$  after the introduction of the indicator into the circulation,  $F_p^*$  the constant rate of collection of arterial plasma,  $Q_p(T)$  the amount of radioactive butanol collected, and  $C_p(T)$  the amount of indicator per unit weight of a sample of brain to which  $P^*$  refers.  $E(T)$  was estimated as described previously (Gjedde et al. 1980).

Whole-brain and regional blood-brain glucose transfer rates were calculated from tissue and arterial blood sample contents of radioactive glucose, assuming no loss of labeled glucose from the brain during the time  $T$  (Gjedde et al. 1980).

$$J_{\text{glc}} = C_p^* F_p^* \frac{C_p(T)}{Q_p(T)} - C_p^{**} F_p^* \left[ \frac{C_p(T)}{Q_p(T)} \frac{V^* C_p^{**}(T)}{C_p^*(T)} \right] \quad (10)$$

where  $J_{\text{glc}}$  represent the unidirectional blood-brain glucose flux per unit weight of brain,  $C_p^*$  the arterial plasma glucose concentration,  $F_p^*$  the plasma flow per unit weight of the sample of brain,  $F_p^{**}$  the volume of plasma collected in the arterial sampling syringe in unit time,  $C_p^*(T)$  and  $C_p^{**}(T)$  the extravascular and total contents of labeled glucose per unit weight of the sample of brain,  $V^*$  the plasma volume in brain,  $Q_p(T)$  the total content of labeled glucose in the arterial sample and  $C_p^{**}(T)$  the concentration of labeled glucose in arterial plasma at the time  $T$ . The plasma volume was measured in separate experiments as described previously (Gjedde et al. 1980).

The extraction fraction of tracer glucose was calculated from rearrangement of equation (9),

$$E_{\text{glc}} = \frac{F_p^* C_p^*(T)}{P^* Q_p(T)} \quad (11)$$

#### General procedure

12 male Wistar rats were divided in 3 groups. Rats in the normal glucose group received no treatment. Rats in the low glucose group received 2.10 kg i.v. insulin neutral (Leo) p. 1 h prior to study. Rats in the high glucose group received 17 mmol/kg D-glucose i.p. 1 h prior to study.

The 300–350 g rats were kept specific-pathogen-free and fed food and water ad libitum. Anaesthesia was induced with ether and the animals were paralyzed with 3 mg/kg<sup>-1</sup> succinylcholine, intubated and ventilated with 15% halothane vaporized in a mixture of nitrogen and oxygen (70:30).

Catheters were placed in the tail artery, both femoral arteries, one femoral vein, and the superior sagittal sinus of the brain. Cannulation of the superior sagittal sinus was performed as described previously (Gjedde et al. 1980).

Arterial blood samples were assayed for pH,  $P_{\text{aO}_2}$  and  $P_{\text{aCO}_2}$  with microelectrodes (Radiometer Copenhagen). The animals were considered to be in respiratory steady-state when  $P_{\text{aCO}_2}$  in samples taken at least 10 min apart agreed within 10%. Arterial blood pressure was continuously recorded with strain gauge transducer.

When the animals were in respiratory steady-state a mixture of 5  $\mu\text{Ci}$  [<sup>14</sup>C]butanol and 20  $\mu\text{Ci}$  [<sup>3</sup>H]glucose in 200  $\mu\text{l}$  Ringer-HEPES buffer solution (Gjedde & Crone 1975) was rapidly injected into the femoral vein. Prior to mixing, the [<sup>3</sup>H]glucose solution was air-dried and resuspended to eliminate all volatile <sup>3</sup>H-activity.

Integration of concentration-time curves of arterial blood were produced mechanically as described previously (Gjedde et al. 1980). Briefly arterial blood was drawn into calibrated glass syringes at known and constant rates during and for 20 s after injection. At the end of 20 s, withdrawal was discontinued and the animal decapitated with rodent guillotine. The brain was rapidly removed and dissected bilaterally into the following regions, weighing approximately 75 mg each: Cerebellar hemisphere, cerebellar vermis, medulla and pons, midbrain, basal

Table 1 General conditions of the three groups of rats used in the present study

Group	Normal glucose (n=5)	High glucose (n=4)	Low glucose (n=4)
MABP (mmHg)*	100±3	102±13	101±8
hct (%)	44±2	48±2	44±
P <sub>a</sub> (mmHg)*	33±2	29±0.4	35±2
P <sub>ao</sub> (mmHg)*	130±15	145±20	147±20
pH†	7.47±0.02	7.41±0.03	7.39±0.02
f <sup>cm</sup> (ml (100 g) min <sup>-1</sup> )	72±4	45±10	65±7

Values indicate mean ± S.E. Number of observations in brackets.

Mean arterial blood pressure \* Arterial hematocrit  
Arterial carbon dioxide tension † Arterial oxygen tension  
Arterial pH ‡ Cerebral plasma flow rate

ganglia, parietal cortex and frontal occipital and temporal lobes.

The blood and tissue samples were rapidly transferred to pre-weighed counting vials containing 1.5 ml of a Soluene (Packard) and isopropanol mixture (1:1). The vials and samples were then re-weighed, blanchied with 0.5 ml 35% hydrogen peroxide and stored overnight at 50°C. The next morning preparation was completed by addition of 20 ml of an Instagel (Packard) and 0.5 N HCl mixture (9:1). The vials were allowed to stabilize at counting temperature for 4 h prior to counting in a Tri-Carb 2425 (Packard) liquid scintillation spectrometer.

Plasma concentrations of glucose were measured in freshly sampled arterial and cerebral venous plasma by the glucose oxidase method (Christensen 1967; Hjelm & De Verdier 1963). Blood concentrations of oxygen were determined by carbon monoxide displacement in a galvanic fuel cell (Lex-O<sub>2</sub>-Con Lexington Instruments). Arterial hematocrit was determined by centrifugation.

Consumption of glucose and oxygen was calculated by multiplying arteriovenous deficits with the whole brain plasma and blood flow rates, respectively.

Preparations of  $n$ -[1-<sup>14</sup>C]butanol, D-[1-<sup>3</sup>H(N)]glucose and D-[1-<sup>3</sup>H(N)]mannitol were obtained from the New England Nuclear Corporation with specific activities of 1.86 mCi/mmol, 18 Ci/mmol and 15–30 Ci/mmol, respectively.

## RESULTS

The average whole brain plasma flow rates of the three groups of rats, arterial blood gases, pH and mean blood pressure are recorded in Table 1. No significant differences were noted between the three groups.

The average plasma volume (measured with mannitol) per unit weight of brain of the three groups of rats were  $1.01 \pm 0.17$  (S.E.,  $n=8$ ),  $0.11$  (S.E.,  $n=4$ ) and  $0.54 \pm 0.03$  (S.E.,  $n=4$ ) (100 g)<sup>-1</sup> respectively in normo-, hyper- and hypoglycemic animals.

The observed arterial glucose and oxygen concentrations and the cerebral metabolic rates of glucose and oxygen are shown in Table 2. Glucose consumption, calculated as  $f^m(C_a^g - C_v^g)$ , was significantly increased in hyperglycemia and markedly reduced in hypoglycemia and markedly reduced in hypoglycemia. Oxygen consumption was similar in the three groups. Oxygen consumption in the normo- and hyperglycemic groups reflects the normal ratio (6 mol/mol) between oxygen and glucose consumption in the brain. In the hypoglycemic group, oxygen consumption exceeded the rate calculated stoichiometrically from glucose consumption, indicating oxidation of other substrate than glucose, as also noted by Norberg & Siev (1976).

The average whole brain extraction fraction and flux of unidirectionally transported glucose of the three groups are shown in Table 3. The relationship between the concentration of glucose in arterial plasma and the transport of glucose from blood to brain in individual animals is shown in Fig. 1. It is evident that the unidirectional blood-brain glucose transfer increases at a declining rate with increasing plasma glucose concentration. This phenomenon, known as saturation, is one of several criteria of facilitated diffusion and forms the basis for the use of the Michaelis-Menten formalism that leads to the maximal transport rate and a half maximal constant. For reasons outlined in theory above, it is meaningless to calculate a regression curve between arterial glucose concentration and blood-brain glucose transfer for the points shown in Fig. 2, because the blood-brain glucose flux is not a function of the arterial plasma concentration alone. It was shown that the glucose metabolic rate can be ignored in the calculation of  $J_{\text{net}}$  when the ratio  $\text{CMR}_{\text{glc}}/[f^m(K_m + C_a^g)]$  does not exceed 0.1 (cf. Table 2). A plot of  $-C_a^g/f^m \ln(1 - E_{\text{glc}})$  against  $f^m \ln(1 - E_{\text{glc}})$  yields  $T_{\text{m}}$  and  $K_m$  (cf. Fig. 3). The points regressed to the linear equation  $y = -5.91x + 5.8$  with a correlation coefficient of 0.7, indicating a  $T_{\text{m}}$  of  $25.8 \pm 3.3$  (S.E.)  $\mu\text{mol}$  (100 g) min<sup>-1</sup> and a  $K_m$  of  $5.9 \pm 1.6$  (S.E.) mmol/l.

The regional variation of  $T_{\text{m}}$  and  $K_m$  was investigated

Table 2 Arterial concentrations and cerebral metabolic rate for oxygen and glucose

mg	$C_{aO_2}$ (mmol l <sup>-1</sup> )	$C_a$ (mmol l <sup>-1</sup> )	$CMR_{O_2}$ ( $\frac{\mu\text{mol}}{100\text{ g min}}$ )	$CMR_{glc}$ ( $\frac{\mu\text{mol}}{100\text{ g min}}$ )	$\frac{CMR_{glc}}{(C_a - C_v) K_m}$
norm glucose	9.1 ± 0.1	10.4 ± 0.9	379 ± 49	67 ± 10	0.0571
hypo glucose	10.4 ± 0	30.8 ± 0.7	433 ± 76	74 ± 8	0.0448
hyper glucose	9.1 ± 0.7	1.8 ± 0.6	313 ± 61	31 ± 6	0.0619

Values indicate mean ± S.E.

$C_{aO_2}$ : arterial oxygen concentration (whole-blood)  $C_a$ : arterial plasma glucose concentration  $CMR_{O_2}$ : whole-brain metabolic rate for oxygen  $CMR_{glc}$ : whole-brain metabolic rate for glucose

brain and indicated homogenous tissue. No grey matter was sampled, however.

## DISCUSSION

The present study revealed a maximal rate of transport of glucose across the blood-brain barrier higher than previous estimates in rat (Brender et al. 1970; Partridge & Oldendorf 1977). The observed apparent affinity constant is only slightly lower than previous estimates in rat (Bachelard et al. 1973; Zinder et al. 1973; Buschleuzer et al. 1970; Partridge & Oldendorf 1977).

### Glucose transport rate

The transport capacity of the blood-brain barrier for glucose is affected by a number of factors that are present not well known. It is not unlikely that the maximal transport capacity is related to the surface area of perfused capillaries which may vary between species, and in a particular animal under various conditions. It is possible, therefore, that the

assumption of constant  $T_{max}$  in the range of plasma glucose concentration from 1.8 to 30 mM is not valid. In particular the question may be raised whether the low blood flow rate ( $f^m$ ) observed in the hyperglycemic rat were not associated with a reduction of the number of perfused capillaries and thus of  $T_{max}$ . However the fact that  $E_{app}$  was not found to be much lower in the hyperglycemic animals than in the normoglycemic speaks against this possibility. An elevation of  $C_a^m$  and a reduction of  $T_{max}$  should both (cf. equation (7)) lead to a decrease of  $E_{app}$ . It is therefore likely that the lower  $f^m$  has exerted the opposite effect on  $E_{app}$  as it would when the number of perfused capillaries (and  $T_{max}$ ) was unchanged and the plasma transit time prolonged.

Other factors which may affect blood-brain glucose transport include anaesthetic agents. They may act directly on the endothelial membranes or indirectly through changes of cerebral blood flow. Thus, Mayman et al. (1964) and Alexander et al.

Table 3 Blood-brain glucose transport in the three groups of rats

Group	$E_{app}$ (%)	$J_{un}$ ( $\mu\text{mol (100 g)}^{-1}\text{ min}^{-1}$ )
Norm glucose	21 ± 1	190 ± 11
Hypo glucose	17 ± 3	205 ± 27
Hyper glucose	37 ± 1	44 ± 13

Values indicate mean ± S.E.

$J_{un}$ : extracted fraction of unidirectionally transported tracer glucose.  $J_{un}$ : blood-brain glucose flux per unit weight of whole brain.

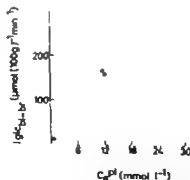


Fig. 2 Relationship between arterial plasma glucose concentration and unidirectional blood-brain glucose flux per unit weight of whole-brain in rats rendered normo-, hypo- or hyperglycemic by administration of glucose or insulin.

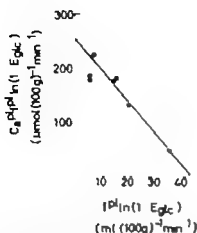


Fig. 3 Estimate of the constants  $T_{max}$  and  $K_m$  for the transport of glucose from blood to brain by means of a double-logarithmic plot based on the mathematical treatment of blood-brain glucose transfer developed in the text

(1975) reported that halothane and pentobarbital may render the blood-brain barrier more permeable to hexoses. In most of the previous studies on rat cited above, anesthesia was achieved by barbiturates, while halothane was used in the present study. For this reason alone, direct comparison with the present study is difficult. Furthermore, the value for maximal glucose transport reported by Pardridge and Oldendorf (1977) was not based on measurement of cerebral blood flow, although this variable was needed to calculate transport rates (Gjedde & Crone 1975).

#### Half saturation constant

The half saturation constant measured in the present study ranks among the lower ones in the literature and was determined with correction for passive diffusion. The determination of the half saturation constant is very sensitive to uncertainties about the exact capillary glucose concentration. Therefore, steady state with respect to unlabeled glucose concentrations is a prerequisite for accurate calculation of the constant. Non-steady state may introduce marked redistribution of unlabeled glucose between blood and brain (Lund Andersen & Christensen 1979).

#### Compartmental analysis

A compartmental analysis of blood-brain glucose transfer and phosphorylation rates following intravenous injections of tracers was performed by Buschiazzo et al. (1970). No account was taken of the average glucose concentration in the cerebral capillaries

and the extracellular glucose concentration was assumed to approach the mean capillary glucose concentration at high glucose levels in blood. Equilibrium concentrations have never been observed in experiments, nor can they be inferred from the symmetrical nature of the glucose carrier. Equation (1) in fact predicts that, provided  $CMR_{glc}$  is constant, the plasma to brain glucose ratio is rather constant at plasma glucose concentrations above a certain level (of the order of 20 mM), falling only when the concentration reached very high levels. For a considerable range of plasma concentrations, the major determinant of the concentration ratio is the  $CMR_{glc}$ .

A kinetic analysis of brain glucose uptake was performed by Pappenheimer & Setchell (1979) in sheep and rabbit. Their analysis was based on the assumption that the difference between capillary and brain glucose concentrations falls exponentially along the capillary, reaching zero at its midpoint, and that the brain extravascular glucose concentrations in the steady state can be represented by the glucose concentration in cerebrospinal fluid. It is suggested in the present analysis that the capillary glucose concentration more likely falls linearly, a rate determined by the glucose consumption in the tissue. It was recently convincingly argued by Lund Andersen (1979) that free glucose is distributed uniformly in brain water and that its concentration reaches no more than half of that in cerebrospinal fluid. Pappenheimer & Setchell, however, obtained values for maximal glucose transport as half saturation constant in the sheep and rabbit, which agree with the results of the present study.

It is important to note that the present method requires that the metabolic rate for glucose in brain be independent of the glucose concentration in blood and brain. Although this was not the case in the present study, it was shown that  $CMR_{glc}$  did not enter into the calculation of the kinetic constants from data from the entire range of concentrations studied.

However, in hypoglycemia, in which low plasma glucose levels directly or indirectly cause  $CMR_{glc}$  to fall, the assumption made here in integrating equation (1), i.e. that  $CMR_{glc}$  be the same at all points along the capillary, may no longer be valid, and equations (6), (7) and (8) therefore no longer valid for the case of hypoglycemia. The weight of this objection depends on the influence of a varied  $CMR_{glc}$  on the mean  $E_{glc}$ . In the hypoglycemic

some animals, the net cerebral glucose extraction fraction is about 25% indicating a fall of unidirectional glucose concentration of the same magnitude during the passage of blood through the cerebral capillaries. If  $\text{CMR}_{\text{glc}}$  decreased by the same amount, i.e. from  $35 \mu\text{mol} (100 \text{ g})^{-1} \text{min}^{-1}$  at the arterial end of the capillary to  $27 \mu\text{mol} (100 \text{ g})^{-1} \text{min}^{-1}$  at the venous end of the capillary (in order to yield an average  $\text{CMR}_{\text{glc}}$  per unit weight of whole brain of  $31 \mu\text{mol} (100 \text{ g})^{-1} \text{min}^{-1}$ ) stepwise integration of equation (5) (or by division of the capillary into equal sections in which  $\text{CMR}_{\text{glc}}$  decreased from one section to the next) predicts that the resulting  $E_{\text{glc}}$  could decrease by less than 1% if the value calculated from equation (7) under the assumption that  $\text{CMR}_{\text{glc}}$  remained constant and equal to the  $\text{CMR}_{\text{glc}}$  present at the arterial end of the capillary. The assumption of constant  $\text{CMR}_{\text{glc}}$  therefore fails to introduce any significant error in the final estimation of  $T_{\text{max}}$  and  $K_{\text{m}}$  from values of  $E_{\text{glc}}$  obtained in the entire range of  $C_{\text{glc}}^{\text{a}}$  values studied.

The present study took account of cerebral blood flow, the cerebral metabolic rate for glucose and the capillary concentrations of labeled and unlabeled glucose. It was a consequence of the model that labeled and unlabeled glucose appeared to be differently unlabeled glucose falling linearly and labeled glucose falling exponentially in the capillary. No account was taken of the possibility that the blood-brain barrier may consist of two or more distinct barriers represented by the basal and additional membranes of the cerebral capillary endothelial cell. The analysis yielded unidirectional constants for the blood-brain transport of glucose, based on a model which included more of the variables that effect the measurement of blood brain glucose transport than have previous studies.

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# LIST OF SYMBOLS

Symbol	Description
$A_{\text{cap}}$	capillary plasma cross-sectional area of single brain capillary ( $\text{cm}^2$ )

$b$	ratio between maximal rate of unidirectional blood-brain glucose transport and capillary plasma volume per unit weight of brain ( $\text{ml ml}^{-1}$ )
$C^{\text{a}}$	total D-glucose concentration in arterial plasma ( $\text{mM}$ )
$C_{\text{glc}}^{\text{a}}(t)$	concentration of labeled D-glucose in arterial plasma as a function of time after introduction into the circulation ( $\text{nCi ml}^{-1}$ )
$C_{\text{glc}}^{\text{a}}(T)$	concentration of labeled D-glucose in arterial plasma at time of decapitation ( $\text{nCi ml}^{-1}$ )
$C_{\text{glc}}^{\text{c}}(t)$	total concentration of glucose in capillary plasma as a function of time spent in the capillary ( $\text{mM}$ )
$C_{\text{glc}}^{\text{c}}(T)$	concentration of labeled D-glucose in capillary plasma as a function of time spent in the capillary ( $\text{nCi ml}^{-1}$ )
$C_{\text{glc}}^{\text{v}}$	total concentration of D-glucose in venous plasma ( $\text{mM}$ )
$C_{\text{glc}}^{\text{v}}(t)$	concentration of labeled D-glucose in venous plasma as a function of time after introduction into the circulation ( $\text{nCi ml}^{-1}$ )
$C$	total concentration of D-glucose in extracellular water in brain ( $\text{mM}$ )
$C_{\text{glc}}^{\text{b}}(T)$	content of labeled D-glucose per unit weight of brain at time of decapitation ( $\text{nCi} (100 \text{ g})^{-1}$ )
$C_{\text{glc}}^{\text{b}}(T)$	extravascular content of labeled D-glucose per unit weight of brain at time of decapitation ( $\text{nCi} (100 \text{ g})^{-1}$ )
$C_{\text{glc}}^{\text{a}}(t)$	concentration of labeled butanol in arterial blood as a function of time after introduction into the circulation ( $\text{nCi ml}^{-1}$ )
$C_{\text{glc}}^{\text{a}}(T)$	concentration of labeled butanol in arterial blood at time of decapitation ( $\text{nCi ml}^{-1}$ )
$C_{\text{glc}}^{\text{b}}(t)$	content of labeled butanol per unit weight of brain as a function of time after introduction into the circulation ( $\text{nCi} (100 \text{ g})^{-1}$ )
$C_{\text{glc}}^{\text{b}}(T)$	content of labeled butanol per unit weight of brain at time of decapitation ( $\text{nCi} (100 \text{ g})^{-1}$ )
$C_{\text{glc}}^{\text{v}}(t)$	concentration of labeled butanol in cerebral mixed venous blood as a function of time after introduction into the circulation ( $\text{nCi ml}^{-1}$ )
$C_{\text{glc}}^{\text{v}}(T)$	content of labeled butanol per unit weight of an organ indicated by subscript at the time of decapitation ( $\text{nCi} (100 \text{ g})^{-1}$ )
$\text{CaO}_2$	concentration of oxygen in arterial blood ( $\text{mmol l}^{-1}$ )
$\text{CMR}_{\text{glc}}$	metabolic rate for glucose per unit weight of brain ( $\mu\text{mol} (100 \text{ g})^{-1} \text{min}^{-1}$ )
$\text{CMR}_{\text{glc}}^{\text{a}}$	metabolic rate of glucose in small section of average single capillary ( $\mu\text{mol min}^{-1}$ )
$\text{CMR}_{\text{O}_2}$	metabolic rate for oxygen per unit weight of brain ( $\mu\text{mol} (100 \text{ g})^{-1} \text{min}^{-1}$ )
$E_{\text{glc}}$	fraction of unidirectionally extracted glucose in brain (ratio)
$E(T)$	cerebral extraction fraction of butanol integrated to time of decapitation (ratio)
$E_{\text{glc}}(T)$	extraction fraction of butanol in organ indi-



cated by subscript  $\equiv$  at time of decapitation (ratio)  
 plasma flow rate per unit weight of brain (ml (100 g) min<sup>-1</sup>)  
 blood flow rate per unit weight of brain (ml (100 g) min<sup>-1</sup>)  
 blood flow rate per unit weight of an organ indicated by subscript "o" (ml (100 g) min<sup>-1</sup>)  
 total plasma flow to organ indicated by subscript o rate of collection of arterial plasma (ml min<sup>-1</sup>)  
 total blood flow to organ indicated by subscript o rate of collection of arterial blood (ml min<sup>-1</sup>)  
 cardiac output (ml kg<sup>-1</sup> min<sup>-1</sup>)  
 hematocrit (ratio)  
 unidirectional blood brain glucose transfer rate per unit weight of brain ( $\mu$ mol (100 g) min<sup>-1</sup>)  
 rate of monoexponential decay of arterial labeled butanol concentration (s)  
 half saturation constant of unidirectional blood-brain D-glucose transfer (mM)  
 brain blood partition coefficient for butanol (ml g)  
 mean arterial blood pressure (mmHg)  
 number of perfused capillaries per unit weight of brain ((100 g)<sup>-1</sup>)  
 rate constant of labeled butanol turn-over in brain (s)<sup>-1</sup>  
 arterial oxygen tension (mmHg)  
 arterial carbon dioxide tension (mmHg)  
 arterial pH  
 permeability surface area product of unidirectional blood brain transfer of solutes (ml (100 g) min<sup>-1</sup>)  
 content of labeled D-glucose in organ indicated by subscript  $\equiv$  at time of decapitation amount of labeled D-glucose collected in arterial syringe (nCi)  
 content of labeled butanol in organ indicated by subscript o at time of decapitation amount of labeled D-glucose collected in arterial syringe (nCi)  
 total amount of labeled butanol given at time  $t=0$  (nCi kg)  
 time after intravenous bolus injection (s)  
 time from intravenous bolus injection to peak of arterial labeled butanol concentration curve (s)  
 mean transit time of plasma in brain (s)  
 time spent in cerebral capillary (s)  
 time of decapitation (s)  
 maximal rate of unidirectional blood-brain glucose transfer per unit weight of brain ( $\mu$ mol (100 g) min<sup>-1</sup>)  
 maximal rate of unidirectional blood-brain glucose transfer in a small section of n e r age single cerebral capillary ( $\mu$ mol min<sup>-1</sup>)  
 volume of plasma in small section of average single cerebral capillary (ml)

V volume of capillary plasma per unit weight of brain (ml (100 g)<sup>-1</sup>)  
 V<sup>m</sup> volume of plasma per unit weight of brain (ml (100 g)<sup>-1</sup>)

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## Ultrastructure of neurosecretosomes sedimented in the nuclear fraction from the posterior pituitary of the rat

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KANERVA, L., TISSARI, A. H., RECHARDT, L. & HERVONEN, A. Ultrastructure of neurosecretosomes sedimented in the nuclear fraction from the posterior pituitary of the rat. *Acta Physiol Scand* 1980, 108: 341-345. Received 29 May 1979. ISSN 0001-6772. Departments of Anatomy and Pharmacology, University of Helsinki, and Department of Biomedical Sciences, University of Tampere, Finland.

Subcellular fractions of the posterior pituitary of the rat were isolated by differential and density gradient centrifugation and microsamples prepared for electron microscopy by  $\text{KMnO}_4$  or glutaraldehyde- $\text{OsO}_4$  fixation. The nuclear fraction (P (1000 g, 7 min), was the main neurosecretosome (NSS) fraction and contained nuclei and mitochondria in addition to NSS (60%). The crude mitochondrial fraction, P (10000 g, 20 min), contained free mitochondria (70%) NSS, unidentified membrane particles, fat droplets, derived from pituitary cells, neurosecretory granules (NSG) and synaptosomes, identified by the presence of synaptic membrane thickening. Fraction P (32000 g, 70 min) contained mainly free NSG while fraction P (118000 g, 30 min) had a high microsome content.

**Key words:** Posterior pituitary, subcellular fractionation, neurosecretosomes, synaptosomes, ultrastructure.

It is known that pinched-off nerve endings (synaptosomes) isolated from the brain sediment in differential centrifugation in the mitochondrial fraction and can be purified partly by centrifugation in density gradients, as a result of which they sediment in lighter subfractions than mitochondria (De Robertis & Rodriguez de Lores Arnaiz 1969; Whittaker 1969). The sedimentation characteristics of synaptosomes are so uniform that attempts to separate them into subclasses according to the transmitter or receptor specificity have met with only limited success. There are a few exceptions to this uniformity. The large mossy fibre synaptosomes from the cerebellum sediment in the nuclear fraction in differential centrifugation (Hayda et al. 1974). Another deviation is seen with synaptosomes from developing brain, which sediment in the mitochondrial fraction but during subfractionation show a wide variation of densities, ranging from the lightest to the heaviest (for ref. see Hervonen et al. 1974; Kanerva et al. 1977).

Several studies have been carried out on the subcellular fractionation of the posterior pituitary mainly using bovine tissue because of the small size of the organ. The results obtained in differential and density gradient centrifugation indicate that pinched-off neurosecretory nerve endings (neurosecretosomes, NSS) from the posterior pituitary have a sedimentation density higher than that of mitochondria and ordinary synaptosomes, though lower than that of nuclei (La Bella et al. 1965; Bindler et al. 1967; Lederis & Livingstone 1970; Vilhardt & Baker 1976; Boer et al. 1976; Vilhardt et al. 1977). By employing a micromethod for the preparation of samples for electron microscopy (Cotman & Flansburg 1970) we used glutaraldehyde-osmium tetroxide and  $\text{KMnO}_4$  fixation to study the fine structure of subcellular fractions from the posterior pituitary of the rat. Our finding was that neurosecretosomes sediment in the nuclear fraction. Non-neurosecretory nerve endings have also been isolated from the posterior pituitary. Pre-

liminary findings have been reported (Kanerva et al 1978)

## MATERIAL AND METHODS

Virgin female and male Sprague-Dawley rats 2-3 months old were used. The subcellular fractionation procedure was that used by Bindler et al (1967) with slight modifications. In each experiment which was repeated several times about 30 rats of either sex were decapitated with a guillotine between 9 and 11 a.m. The posterior pituitary was immediately dissected out under a stereomicroscope (40 $\times$ ) at 4°C and placed in 0.32 M sucrose in an ice bath. Pooled tissues were homogenized in 9 vol of 0.3 M sucrose in a Teflon/glass homogenizer with a clearance of 0.25 mm (Kontes) by 10 strokes at 1000 rpm. The cylinder was rinsed 4 times with the same volume of 0.32 M sucrose. The homogenate and washings were combined and centrifuged differentially at 1000 $\times$ g for 7 min (fraction P) at 10000 $\times$ g for 70 min (fraction P) at 39000 $\times$ g for 20 min (fraction P<sub>2</sub>) and at 118000 $\times$ g for 30 min (fraction P<sub>3</sub>) leaving a particle-free supernatant, S<sub>4</sub>. Fraction P was washed once with 1 ml of 0.3 M sucrose and the washing combined with supernatant S<sub>4</sub>. In some expts fraction P was suspended in 0.32 M sucrose and layered over a density gradient consisting of 1 ml of 0.174, 1.45 and 1.16 M sucrose and centrifuged at 53000 $\times$ g for 170 min. Subfractions A, B and C sedimenting above 1.16, 1.45 and 1.74 M sucrose respectively were collected by vacuum aspiration in each case including half of the clear zone between the layers diluted with water to a sucrose concentration of 0.53 M allowed to stand for 70 min and centrifuged at 40000 $\times$ g for 30 min. Differential and density gradient centrifugations were performed in a Spinco L 50 or L 5-50 Beckman ultracentrifuge at 4°C using an SW 50.1 or SW 39 L rotor. All g values were calculated at R<sub>0</sub>. The pellets obtained from differential and density gradient centrifugations were suspended in Krebs bicarbonate solution, pH 7.4 (KRB). After 20 min at 0°C aliquots were removed for electron microscopy and occasionally for protein assay (Lowry et al 1951). In some expts the incubation of the P suspension was continued at 37°C and 95% O<sub>2</sub> + 5% CO<sub>2</sub> After 8 min of prewarming 100 µg/ml 5-hydroxydopamine (H 83/35 Hassle Göteborg) 10<sup>-4</sup> M pargyline HCl (Eutonyl Abbott Chicago Ill) and 0.1 mg/ml ascorbic acid (final concentrations) were added and the incubation continued for 15 or 30 min.

Samples for electron microscopy were prepared by the micro-method of Colman & Flansburg (1970). 100-200 µl aliquots of the suspension (about 0.1 mg of protein) were transferred to siliconized bottleneck Beem capsules (Ted Pella, Los Angeles Cal) in an ice bath and fixed as suspension or pellet for electron microscopy. The samples were sedimented by centrifuging them in an SW 5.1 or SW 27 rotor equipped with suitable adapters at 40000 $\times$ g for 30 min. P<sub>2</sub> however was centrifuged for 90 min. The following fixatives were used (all concentrations final): 1) 3% glutaraldehyde in 0.1 M phosphate buffer or 0.05 M cacodylate buffer, pH 7.4 as a suspension for 15 min or as a pellet for 1 h 2) 3% KMnO<sub>4</sub> in 0.1 M phosphate buffer, pH 7.0 as a suspension for 15 min

(Richardson 1966). The samples fixed with glutaraldehyde were postfixed in phosphate-buffered 1% OsO<sub>4</sub> at pH 7 for 1-2 h. The specimens were then dehydrated in grad ethanol and embedded in Epon-Araldite. The sections fixed in KMnO<sub>4</sub> were viewed and photographed stained while the sections fixed in glutaraldehyde were stained on grids with lead citrate and uranyl acetate. A Philips EM 300 electron microscope operated at 60 kV was used.

The estimations of relative particle areas were according to the principle laid down by Leal (1962). A grid consisting of 13 parallel lines was placed over electron micrographs. 70 electron micrographs taken without any selection from each fraction at magnification (x8700). Micrographs were taken from the various regions of the pellet to ensure that they were representative.

## RESULTS

The nuclear fraction P (1000 g $\times$ 7 min Fig 1) contained in order of frequency (relative particle area) NSS (about 60%) nuclei (15%) and heterogeneous mixture of free mitochondria (10-15% of fraction P comprised small numbers of droplets from pituitary free neurosecretory granules (NSG) pinched-off nerve terminals identified by the presence of synaptic membrane thickenings (synaptosomes) fragments of blood vesicles and collagen fibres and unidentified membrane fragments.

The neurosecretosomes showed the same structural variations as those seen in tissue sections (for ref. see Baumgarten et al 1977). Their contents (Fig. 1) varied from complete filling with electron dense NSG to a mixture of NSG and small vesicles and solitary NSG with abundant small vesicles. NSS generally had a diameter of over 1 µm but both smaller and very large NSS were detected.

After glutaraldehyde-osmium tetroxide fixation the NSG were filled with electron-dense material (see Fig. 1). Sometimes the granules appeared empty because of a probable extraction of material during the preparation procedure. The NSG diameters varied in diameter between 100 nm and 200 nm with a mean diameter of about 160 nm (as calculated from 100 NSG). The NSG were covered by a unit membrane which was best visualized after KMnO<sub>4</sub> fixation. This trilaminar structure identical in the NSG microvesicles (MIV) and the normal outer membrane. Other organelles in the fraction were the small mitochondria, neurotubuli, vacuoles and some multilamellated bodies. NSS with distinctive features were also found.



Fig. 1. Nuclear fraction P after 3%  $\text{KMnO}_4$  fixation in suspension. General view of the area with large number of well preserved pinched-off neurosecretory endings (neurosecretosomes) containing neurosecretory vesicles (arrow) and microvesicles (arrow head). The cytoplasm has leaked out from some neurosecretosomes on the right (star), giving the neurosecretosomes light background. 20175

After  $\text{KMnO}_4$  fixation (Fig. 1) the core of the NSG consisted of a reticular substructure: no dense core was observed. As was the case after glutaraldehyde-osmium tetroxide fixation this reticular substructure was missing in some granules, indicating leakage of the protein-peptide complex during the preparation procedure. The MV were agranular after the use of this fixative.

The crude-mitochondrial P fraction ( $10000 \times 20 \text{ nm}$ ) contained (in order of frequency) free mitochondria (70%), NSS (15%), unidentified membrane particles (10%), fat droplets derived from phagocytes, NSG and synaptosomes. The

nerve endings most frequently detected in this fraction, too, were those of the neurosecretory type which contained NSG and clear MV 30 to 60 nm in diameter. A small number of nerve endings were detected without NSG but containing synaptic membrane thickenings, i.e. synaptosomes. These were divided into two groups: 1) monoaminergic (MA) characterized by the presence of small dense-cored vesicles after incubation with 5-hydroxydopamine and fixation with  $\text{KMnO}_4$ , and 2) non-monoaminergic (probably cholinergic) characterized by the presence of clear synaptic vesicles (data not shown). After density gradient centrifuga-

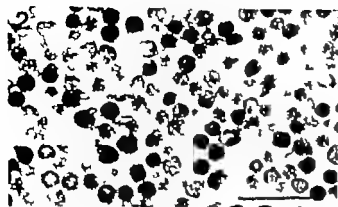


Fig. 2 Fraction P. Glutaraldehyde-OsO<sub>4</sub> fixation. A relatively pure fraction of neurosecretory granules.  $\times 30\,500$ .

tion of fraction P<sub>2</sub> NSS were found in subfractions B and C sedimenting above 1.45 and 1.74 M sucrose respectively.

Fraction P (37 900 g  $\times$  70 min) contained an abundance of free NSG (Fig. 2), small membrane fragments and MV. Fraction P<sub>1</sub> (118 000 g  $\times$  30 min) had a high content of small membrane fragments and MV together with a few NSG.

## DISCUSSION

La Bella et al. (1965) in their first study divided homogenates of the bovine posterior pituitary into 4 fractions sedimenting at 15 000, 63 000, 255 000 and 1 020 000 g-min. As shown by electron microscopy NSS sedimented in the 63 000 g-min fraction and NSG in the 255 000 g-min fraction. In a subsequent study (Bindler et al. 1967) these authors divided the homogenates into 6 fractions sedimenting at 7 000, 23 300, 41 000, 164 000, 658 000 and 3 550 000 g-min. Here NSS sedimented in the 23 300 g-min fraction and NSG in the 658 000 g-min fraction. When the NSS fraction was centrifuged in a density gradient consisting of 1.16, 1.45 and 1.74 M sucrose NSS were found above all the sucrose concentrations. Vilhardt & coworkers (Vilhardt & Baker 1976; Vilhardt et al. 1977) also working with the bovine posterior pituitary separated them into fractions sedimenting at 17 000, 45 000, 75 000, 390 000 and 6 000 000 g-min and characterized the fractions by hormonal and enzymatic markers. They used electron microscopy to verify that NSS sediment in the 45 000 g-min fraction and after density gradient centrifugation at the region of 1.4–1.5 M sucrose and that NSG sediment in the 390 000 g-min fraction. Leders & Livingstone (1970) studied the sub-

fractions of the crude mitochondrial fraction obtained from the posterior pituitary of the rabbit and found that nerve endings have a wide variation in densities ranging from 1.0 to 1.7 M sucrose. Rat posterior pituitaries have recently been investigated by Boer et al. (1976) who prepared subcellular suspensions by a microdispersion method and fractionated them directly in a Ficoll density gradient. NSS sedimented mainly at the 14/22% Ficoll interface phase. In contrast to brain synaptosomes, which sediment at the 7.5/13% Ficoll interface phase (Gossett et al. 1971).

It is clear from the studies referred to above that NSS from the posterior pituitary have a sedimentation density higher than that of synaptosomes. In none of these studies, however, was NSS found in the heaviest nuclear fraction, a finding in the present study. Other completely new findings were non neurosecretory nerve endings, identified by synaptic junctions, in subcellular fractions from the posterior pituitary. These had previously only been found in slices (Baumgarten et al. 1972). One possible reason why neurosecretosomes in the present study sedimented in the 7 000 g-min fraction might be the difference in species. Fractions of different centrifugation from the rat posterior pituitary have not been studied previously. Another possible explanation could be the immediate processing after death, which resulted in good preservation of the electron-dense contents of NSG as seen in the preparations fixed by glutaraldehyde-osmium tetroxide (Fig. 2). A time delay after death might cause loss of some constituents and a decrease in sedimentation density. Indeed Boer & Van Rhee (1976) have found that after water deprivation and a decrease in vasopressin content there is a change in the sedimentation of NSS from the 14/22% Ficoll interface to the 10/14% Ficoll interface.

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# Muscarinic autoreceptor regulates acetylcholine release in rat hippocampus: in vitro evidence

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Release of  $^3\text{H}$  ACh from isolated nerve endings of rat hippocampus was evoked by incubation in Krebs-Ringer buffer containing 25 or 35 mM potassium. The release was  $\text{Ca}^{2+}$ -dependent and could be inhibited by  $\text{Mg}^{2+}$  (30 mM). The muscarinic antagonist atropine ( $10^{-6}$ – $10^{-4}$  M) enhanced  $^3\text{H}$  ACh-release. The muscarinic agonist carbachol ( $10^{-10}$ – $10^{-6}$  M) inhibited  $^3\text{H}$  ACh release via interaction with muscarinic receptors; this effect could be blocked by atropine ( $10^{-4}$  M). The presence of the feed-back regulation of  $^3\text{H}$  ACh release in a cell free preparation provides further evidence that the presynaptic regulation is exerted by muscarinic autoreceptors localized on the cholinergic nerve ending itself. The feed back inhibition of the  $^3\text{H}$ -ACh release does not require the presence of intact neurons or axon terminals. Tetraethanol ( $2.5 \cdot 10^{-4}$  M) does not affect the above results.

**Key words:** Acetylcholine, acetylcholine release, atropine, autoreceptor, presynaptic, synaptosomes.

The importance of presynaptic receptors in the regulation of neurotransmitter release is well documented (cf. Stjärne 1975; Starke *et al.* 1977). Involvement of muscarinic acetylcholine receptors in regulation of acetylcholine (ACh) release from cerebral cortex *in vivo* (Mitchell 1963; Szerb 1964; Jalar & Szerb 1969) and *in vitro* (Polak & Meerums 1966; Molezzer & Polak 1970; Polak 1971; Bourdais *et al.* 1974; Kato *et al.* 1975) has been demonstrated more than a decade ago. Muscarinic regulation of ACh release at the periphery has also been shown (Rosenbaum 1956; Kriebinger & Wagner 1975; Sawyrik & Panayiotidis 1977). Detailed studies on electrically evoked release of ACh from striatal, hippocampal and cortical slices by Szerb and colleagues showed that acetylcholinesterase inhibitors lower the release of  $^3\text{H}$ -ACh (Bourdais *et al.* 1974) where muscarinic antagonists restore or enhance ACh release (Szerb & Sonogyi 1973). Furthermore it was shown that septal lesion which destroys cholinergic afferents to the hippocampus leads to decreased release of  $^3\text{H}$  ACh from hippocampal slices of lesioned animals (Szerb 1977; Szerb *et al.* 1977). However in binding studies with the potent muscarinic antagonist,  $^3\text{H}$ -quinuclidinylbenzilate (QNB), Yamamura & Snyder (1974) were unable

to detect any loss of muscarinic receptor sites in lesioned hippocampus. Thus there is an apparent contradiction between the results indicating the presence of a muscarinic presynaptic receptor that regulates the release of ACh from hippocampus and between the ability of septal lesions to decrease ACh release but not to diminish the number of muscarinic binding sites. Several possible explanations were offered (Szerb 1977) to resolve this contradiction. (a) muscarinic regulation of ACh release is accomplished through interneurons, thus the number of receptors is not reduced by the lesion which only destroys the afferents (b) the antagonist,  $^3\text{H}$ -QNB has so low affinity for the presynaptic receptor that the concentrations used in the binding studies (1 nM) though sufficient to label the postsynaptic muscarinic receptor (Yamamura & Snyder 1974) did not label the presynaptic receptor. Consequently loss of the latter upon the lesion escaped detection.

Whether or not the muscarinic receptors which participate in the feed-back regulation of ACh release in the hippocampus are localized on the cholinergic nerve endings cannot be concluded from the studies carried out in tissue slices as the presence of interneurons can not be excluded in

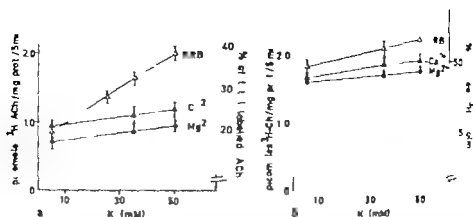


Fig. 1a Dependence of H-ACh release on K<sup>+</sup> concentration in the medium. The osmolality was held constant by decreasing Na<sup>+</sup> concentration to the same extent as K<sup>+</sup> was increased. The KRB was composed as described in Experimental Procedures. —Ca<sup>2+</sup> —Δ— did not contain Ca<sup>2+</sup> and was supplemented with 0.3 mM EGTA. Mg<sup>2+</sup> —●— contained 20 mM Mg<sup>2+</sup> instead of 1 mM. The data are expressed as means  $\pm$  S.D. of triplicate determinations. The experiment was carried out 3 times.

Fig. 1b Dependence of release of H-choline on K<sup>+</sup> concentration in the medium. Conditions were as in Fig. 1a.

such systems. Polak (1971) on the basis of experiments on ACh release from slices in the presence of tetrodotoxin (Molenaar & Polak 1970) suggested that the muscarinic receptors that regulate ACh release are localized on cholinergic nerve terminals. In the present study we addressed the question whether in a cell-free system of nerve endings from rat hippocampus ACh release can still be regulated via a muscarinic receptor. The studies carried out on dilute suspension of synaptosomes in the presence of tetrodotoxin lend further support to the idea that feed-back regulation of ACh release is exerted by muscarinic autoreceptors localized on the cholinergic nerve ending itself.

## EXPERIMENTAL PROCEDURES

### Preparation of nerve ending

5 male Sprague Dawley rats (150–180 g) were decapitated and the hippocampi dissected and placed in ice-cold sucrose medium 0.37 M Tris/HCl 5 mM pH=7.4 and EDTA 40  $\mu$ M. Preparation of P pellet enriched in nerve endings and mitochondria followed the procedure described by Sparagli et al. (1977). In brief 70% (w/w) homogenates were prepared by homogenization at 395 rpm in a loose fitting glass-Teflon homogenizer. After 5 up-down strokes the homogenate was allowed to stand on ice for 5 min before repeating the homogenization. The homogenate was diluted to yield a 10<sup>-6</sup> solution and centrifuged at 1000  $\times$  g for 5 min. The supernatant was further centrifuged at 15000  $\times$  g for 12 min, and the resulting pellet was rinsed twice. The experiments were carried out using the P fraction. (Experiments with synaptosomes prepared according to Jones & Matus (1974) gave the same results.

However the yield of synaptosome preparations was low and the obtained nerve endings were often 'leaky'.

Labelling of nerve endings (4 mg/ml) with [<sup>3</sup>H]-choline (total concentration 8  $\mu$ M H-choline 0.3  $\mu$ M) as carried out according to Sen et al. (1976) in Krebs-Ringer buffer (NaCl 138 mM KCl 5.3 mM MgCl<sub>2</sub> 1 mM NaH<sub>2</sub>PO<sub>4</sub> 1 mM NaHCO<sub>3</sub> 11 mM glucose 10 mM NaOOCCH<sub>3</sub> 1 mM and CaCl<sub>2</sub> 1 mM) for 30 min at 37°C under constant bubbling with 95% O<sub>2</sub>–5% CO<sub>2</sub> gas mixture. At the end of incubation the suspension was centrifuged (400 g for 1 min at 4°C) and the pellet was washed 3  $\times$  5 ml cold Krebs-Ringer buffer supplemented with physostigmine (1 mM).

### Release of H-acetylcholine

The H-choline labelled pellet was resuspended to yield 1 mg/ml protein concentration in ice-cold Krebs-Ringer buffer with physostigmine (0.1 mM). The release was studied at 37°C for 5 min in glass centrifuge tubes containing the drugs studied and K<sup>+</sup> at 5–15 mM or 35 mM concentration. (Increase in K<sup>+</sup> concentration was compensated for by reducing the Na<sup>+</sup> concentration.) Incubation was carried out in a final volume of 100  $\mu$ l containing 100–150  $\mu$ g protein. All release experiments were carried out in the presence of 3-hemicholinium (HC11) (1  $\mu$ M) and tetrodotoxin (TTX) (1.5  $\mu$ M). [<sup>3</sup>H]-ACh and [<sup>3</sup>H]-choline were separated by high voltage (3000 V) paper electrophoresis according to Hildebrand et al. (1974) using triethylammonium marker as described by Lohr & Conzolo (1974). After the run the paper strips were cut into 0.5 cm wide pieces and placed in H<sub>2</sub>O (1 ml) overnight before addition of 10 ml scintillation fluid (Lumac Systems AG Basel). The samples were counted in a Beckman scintillation spectrometer. Protein concentration was determined by the method of Lowry et al. (1951).

Tritium labelled choline (methyl-H-choline) chloride (31 Ci/mmol) and H-acetylcholine chloride (10 Ci/mmol) were purchased from Amersham Pharmacia Biotech.

Table 1. Uptake and release of H-choline and the release of  $^3\text{H}$  ACh from rat hippocampal synaptosomes  
 For experimental details, see Experimental Procedures. The amount of protein present during labelling with  $^3\text{H}$ -choline is 3.5  $\mu\text{g}$ . Data from other experiments agree within 15%

	Picomoles/mg protein	Picomoles
H-choline present in the uptake medium (5 ml)		1 540
H-choline uptake into the synaptosomes	1.5	231
H-ACh in the synaptosomes	5.5	102
H-choline in the synaptosomes	3.8	70
H-choline derivatives (phosphatidylcholine etc.)		
that did not migrate with authentic $^3\text{H}$ -choline	3.2	59
total $^3\text{H}$ ACh release (5 mM K <sup>+</sup> )	0.9	17
total $^3\text{H}$ -ACh release (35 mM K <sup>+</sup> )	1.5	28
total $^3\text{H}$ -choline release (5 mM K <sup>+</sup> )	1.9	35
total $^3\text{H}$ -choline release (35 mM K <sup>+</sup> )	2.1	39

Sweden. England Tetrodotoxin was bought from Aldrich AG, Stockholm, and ouabain from EGA G, Steinheim, West Germany; all other reagents were of the highest quality. The Sigma Chemical Co., St. Louis, Miss., USA solutions are made up daily.

## RESULTS

Depolarizing concentrations of K<sup>+</sup> evoked release of  $^3\text{H}$ -ACh in the presence of extracellular  $\text{Ca}^{2+}$  (Fig. 1). Potassium (50 mM) doubled the release observed at K<sup>+</sup> (5 mM). The latter release may be termed basal or resting release while the release observed in the presence of higher than 5 mM K<sup>+</sup> concentrations will be referred to as evoked release. Omission of  $\text{Ca}^{2+}$  concomitant with application of EGTA (0.3 mM) or application of high  $\text{K}^{+}$  concentrations (20 mM) abolished most of the

high  $\text{K}^{+}$  evoked release without effecting the basal release (Fig. 1a). The resting release or leakage during 5 min at 37°C accounted for ~70% of the total labelled H-ACh (cf. Table 1). Though 50 mM K<sup>+</sup> produced larger release of H-ACh and H-choline than did 35 mM K<sup>+</sup> for studies on the effect of muscarinic ligands on the release this concentration (50 mM) was not suitable. At K<sup>+</sup> concentrations above 35 mM the responsiveness of the system towards atropine and carbachol fell rapidly. Thus most of the experiments were carried out at 5, 25 or 35 mM K<sup>+</sup> concentrations. (The release of  $^3\text{H}$ -choline was much less sensitive to depolarizing concentrations of K<sup>+</sup> or changes in  $\text{Ca}^{2+}$  concentrations (Fig. 1b)).

Electrophoretic separation of the released material was obligatory since  $^3\text{H}$  ACh represented a

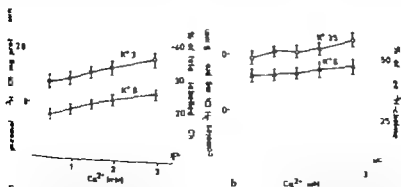


Fig. 2. Dependence of H-ACh release on extracellular  $\text{Ca}^{2+}$  concentrations in the presence of K<sup>+</sup> (5 mM).  $\Delta$ - $\Delta$  K<sup>+</sup> (35 mM)  $\circ$ - $\circ$  The data are expressed as means  $\pm$  S.D. of triplicate determinations. The experiments were carried out 3 times. Fig. 2b. Dependence of  $^3\text{H}$ -choline release on extracellular  $\text{Ca}^{2+}$  concentrations. Conditions were as in Fig. 2a.

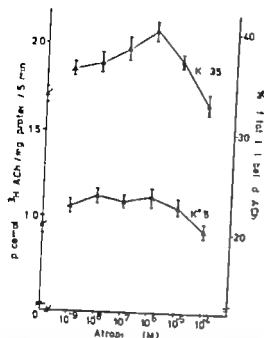


Fig. 3 The effect of various atropine concentrations on the basal ( $5 \text{ mM K}$ )  $\Delta$ - $\Delta$  and evoked ( $35 \text{ mM K}$ )  $\blacktriangle$ - $\blacktriangle$  release of  $^3\text{H}$  ACh. The data represent means  $\pm$  S.D. of quadruplicate determination. The increase in release of  $^3\text{H}$  ACh in the presence of atropine concentrations was significantly ( $p < 0.1$ ,  $p < 0.05$ ) different from the control value at the same  $\text{K}^+$  concentration.

different portion of  $^3\text{H}$ -labelled material released in  $5 \text{ mM K}^+$  vs in  $35 \text{ mM K}^+$  containing medium.

Both basal and evoked release of  $^3\text{H}$  ACh were dependent on the extracellular  $\text{Ca}^{2+}$  concentration (Fig. 2A). The release of  $^3\text{H}$ -choline showed a much smaller dependency on the extracellular  $\text{Ca}^{2+}$  concentration (Fig. 2B).

Atropine in a dose-dependent manner increased release of  $^3\text{H}$  ACh at  $5$  and  $35 \text{ mM K}^+$  concentrations (Fig. 3). Enhancement of the evoked release ( $35 \text{ mM K}^+$ ) was much greater than that of the basal release.

To test the assumption that this finding reflects that the more released ACh is present the more apparent the effect of atropine is, experiments were carried out at different  $\text{Ca}^{2+}$  concentrations.

Fig. 4 shows that the efficacy of atropine, defined as release in the presence minus release in the absence of atropine ( $10^{-6} \text{ M}$ ), is greatly increased with increasing  $\text{Ca}^{2+}$  concentrations. In the case of evoked release, the efficacy of atropine in enhancing basal  $^3\text{H}$  ACh release increased much less with increasing  $\text{Ca}^{2+}$  concentrations.

Atropine also enhanced the release of  $^3\text{H}$ -choline

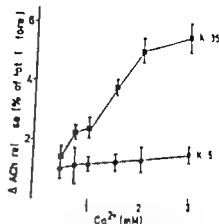


Fig. 4  $\text{Ca}^{2+}$ -dependence of the efficacy of atropine enhancing  $^3\text{H}$  ACh release. ACh release in the presence of atropine ( $10^{-6} \text{ M}$ ) at  $5 \text{ mM K}^+$   $\circ$ - $\circ$  and  $35 \text{ mM K}^+$   $\blacksquare$ - $\blacksquare$  difference in  $^3\text{H}$  ACh release in the presence and absence of atropine ( $10^{-6} \text{ M}$ ) at  $5 \text{ mM K}^+$   $\circ$ - $\circ$  and  $35 \text{ mM K}^+$   $\blacksquare$ - $\blacksquare$ .

with  $10$ - $25\%$  at both  $5$  and  $35 \text{ mM K}^+$  concentrations (data not shown).

The release of  $^3\text{H}$  ACh and the effects of atropine on  $^3\text{H}$  ACh release were not influenced by addition of tetrodotoxin ( $2.5 \mu\text{M}$ ) or by addition or omission of 3-hemicholinium ( $10 \mu\text{M}$ ).

To examine the effects of a muscarinic agonist on  $^3\text{H}$  ACh release the experiments were carried out in the presence of carbachol. d-Tubocurarine (dTC) ( $10^{-5} \text{ M}$ ), a nicotinic antagonist, was present to block nicotinic actions of carbachol. d-Tubocurarine itself had no effect on the basal or evoked release (Table 2). Carbachol ( $10^{-5} \text{ M}$ ) in the presence of dTC ( $10^{-5} \text{ M}$ ) inhibited both basal and evoked release of  $^3\text{H}$  ACh (Table 1).

The weak inhibitory effects of carbachol on  $^3\text{H}$  ACh release could be relieved by  $10^{-6} \text{ M}$  atropine (Fig. 5). At  $1 \text{ mM}$  carbachol concentration  $10^{-6} \text{ M}$  atropine blocked the inhibition on  $^3\text{H}$  ACh release.

Several other drugs were tested in this release system. The muscarinic agonists oxotremorine ( $10^{-5} \text{ M}$ ), methacholine ( $10^{-5} \text{ M}$ ) were very weak inhibitors of evoked release of  $^3\text{H}$  ACh in the presence of  $0.2 \text{ mM}$  physostigmine. The inhibitory effect of muscarinic agonists could be observed easier in the absence of any cholinesterase inhibitor (physostigmine or Sarin) but the data showed much greater variability. The cholinesterase inhibitors physostigmine ( $0.2 \text{ mM}$ ) and Sarin ( $0.1 \text{ mM}$ ) had inhibitory effects on both basal and evoked release (Table 2).

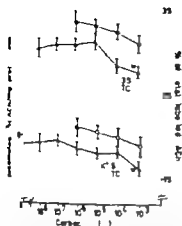


Fig. 1. Influence of cholinergic drugs: carbachol, atropine and *d*-tubocurarine (dTC) on H-ACh release. *d*-tubocurarine  $10^{-6}$  M was present in all expts. depicted on Figure 1. Atropine, when added, was present at  $10^{-6}$  M.  $p < 0.01$  significantly different from control in carbachol present at the same K<sup>+</sup> concentrations.

## DISCUSSION

Release of acetylcholine from hippocampus by muscarinic receptors has been well documented (cf. Szerb 1977). Whether this feed-back inhibition of the release involved neuronal loops or receptors located on the cholinergic nerve endings itself was, however, controversial.

It seemed thus desirable to examine the presence of the muscarinic feed-back regulation in a cell-free preparation of nerve endings from the hippocampus. As synaptosomes do not contain intact neurons or neuronal loops and drugs can reach the nerve endings freely (Maddipati et al. 1975).

Release of the neurotransmitter was evoked by polarizing concentrations of K<sup>+</sup> (25 or 35 mM) or by electrical field-stimulation. K<sup>+</sup> acts directly on the nerve endings and permits simultaneous use of tetrodotoxin (2.5  $\mu$ M) to ensure that spike conduction in neuronal loops is not responsible for the observed feed-back regulation.

As shown in Figs. 1 and 2, depolarizing concentrations of K<sup>+</sup> (25 or 35 mM) release  $^3$ H-ACh and  $^3$ H-choline in a Ca<sup>2+</sup>-dependent manner from the nerve endings. Thus the system fulfils requirements for a physiological release system (Electrical stimulation 10–60 Hz, 20 ms duration for 5 min) or carbachol ( $10^{-6}$ – $10^{-5}$  M) can also be used to release  $^3$ H-ACh in this system.) As shown in Figs. 1

and 2, both H-ACh and  $^3$ H-choline were released at 5 and 35 mM K<sup>+</sup> concentrations, however release of H-choline showed a lower degree of dependency on the K<sup>+</sup> concentrations and on the Ca<sup>2+</sup> concentrations. The fraction of total labelled  $^3$ H-ACh that was released during a 5 min incubation with elevated K<sup>+</sup> concentration is 20–40%. This figure is rather high when compared to fractional release from intact nerves or organs; however it should be noted that (a) we do not know how much of the total ACh is released since we follow a rapidly turning over labelled pool of H-ACh and (b) that it is assumed that the nerve endings do not regenerate normal membrane potential after K<sup>+</sup> depolarization. The fractional release of ACh from *Torpedo ocellata* (Michaelson et al. 1979) and from hippocampal synaptosomes (Nemeth & Cooper 1979) was of similar magnitude (basal release ~20%).

Atropine enhanced the evoked (K<sup>+</sup> 35 mM) release of H-ACh. This effect on  $^3$ H-ACh release clearly indicates the presence of muscarinic receptors on nerve endings from hippocampus. The half maximal effect was attained between  $10^{-8}$ – $10^{-6}$  M concentrations which are well comparable to the value of binding constants of H-atropine to homogenates of rat brain (Alberts & Bartfalvi 1976). Szerb (1977) has found that the half maximal atropine effect in enhancing  $^3$ H-ACh release from slices was

Table 2. The effect of various drugs on H-ACh release from synaptosomes from rat hippocampus

Drug	Percentage of basal release	
	K <sup>+</sup> = 5 mM	K <sup>+</sup> = 35 mM
No drug present	100 ± 17	157 ± 10
Atropine (1 $\mu$ M)	106 ± 14	182 ± 17*
Physostigmine (200 $\mu$ M)	71 ± 11	114 ± 15
Sarin* (100 $\mu$ M)	66 ± 15	117 ± 11
Tetrodotoxin (2.5 $\mu$ M)	104 ± 15	164 ± 21
<i>d</i> -Tubocurarine (10 $\mu$ M)	105 ± 9	179 ± 19
Carbachol (1 mM)	91 ± 17	124 ± 13*
Carbachol (1 mM) + <i>d</i> -tubocurarine (10 $\mu$ M)	67 ± 11	125 ± 20*
Carbachol (1 mM) + <i>d</i> -tubocurarine (10 $\mu$ M) + atropine (10 $\mu$ M)	114 ± 19	165 ± 21

\*From single experiment carried out in triplicates.  $p < 0.01$  is significantly different from 'no drug present' at the same K<sup>+</sup> concentration. All data represent the mean ± S.D. for 6 independent experimental points. 100% basal release of H-ACh corresponds to  $0.92 \pm 0.14$  pmoles  $^3$ H-ACh/mg protein  $\times 5$  min.

about 17 nM. The efficacy of atropine increased with the increased concentrations of <sup>3</sup>H ACh that were present. Thus the efficacy of atropine was higher at higher Ca<sup>2+</sup> concentration (Fig. 4).

A drawback of the system used is that release of <sup>3</sup>H ACh was measured rather than release of endogenous ACh. Since it is known that ACh occurs in several pools in the nerveendings (Simon et al 1976) and in the superior cervical ganglion (Birks & Macintosh 1961) it is not possible to draw any conclusion concerning the total release of ACh on the basis of data on release of <sup>3</sup>H ACh. Nevertheless the existence if not the extent of any regulatory mechanisms governing release of ACh can be established by the system used. Our results yield evidence in support of the hypothesis by Polak (1971) that feed-back regulation of acetylcholine release from hippocampus is exerted via muscarinic presynaptic receptors which are localized on the cholinergic nerve ending and therefore act as autoreceptors.

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about 17 nM. The efficacy of atropine increased with the increased concentrations of  $^3\text{H}$  ACh that were present. Thus the efficacy of atropine was higher at higher Ca concentration (Fig. 4).

A drawback of the system used is that release of  $^3\text{H}$  ACh was measured rather than release of endogenous ACh. Since it is known that ACh occurs in several pools in the nerve endings (Simon et al 1976) and in the superior cervical ganglion (Birks & MacIntosh 1961) it is not possible to draw any conclusion concerning the total release of ACh on the basis of data on release of  $^3\text{H}$  ACh. Nevertheless the existence if not the extent of any regulatory mechanisms governing release of ACh can be established by the system used. Our results yield evidence in support of the hypothesis by Polak (1971) that feed back regulation of acetylcholine release from hippocampus is exerted via muscarinic presynaptic receptors which are localized on the cholinergic nerve ending and therefore act as autoreceptors.

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# Brain extracellular space during spreading depression and ischemia

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HANSEN S. J. & OLSEN C. E., Brain extracellular space during spreading depression and ischemia. *Acta Physiol Scand* 1980; 108: 355-365. Received 23 June 1979. ISSN 0001-6772. Institute of Medical Physiology, Department A, University of Copenhagen and Chemistry Department, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

The change of extracellular space volume of rat brain cortex during ischemia and cortical spreading depression (CSD) (Leão 1944) was evaluated by a new method. The cortical surface was irrigated with isotonic CSF containing the extracellular markers 90 mM choline or 50 mM trimethyltin(hydroxymethyl)trimethyl ammonium ion (N-TRIS), and their extracellular concentrations were monitored by ion-selective microelectrodes. When steady-state for the concentration of these markers was attained, CSD evoked a reversible increase of the concentration of the markers, indicating shrinkage of the interstitial volume of distribution. During ischemia an initial slow rate of concentration increase was observed, followed a few minutes later by rapid increase concomitant with the sharp rise in extracellular potassium concentration. During CSD and ischemia the maximal increases of choline and N-TRIS concentration reflected a shrinkage of the extracellular space amounting to about 40% of the initial volume.

**Key words:** Brain extracellular space, ion-selective microelectrodes, choline, cortical spreading depression, ischemia.

The basic composition of brain extracellular space is subject to significant changes during ischemia and cortical spreading depression (CSD). The concentrations of sodium, chloride and calcium decrease (Dora & Zeuthen 1976; Hansen 1979; Kraig & Nicholson 1978; Nicholson & Kraig 1975) while its potassium concentration undergoes rapid elevation (Dora & Zeuthen 1976; Hansen 1977, 1979; Nicholson & Kraig 1975; Vyskočil *et al.* 1972). Absolute movements of ions between the extra- and intracellular spaces cannot be calculated without knowledge of the variation of the volume of the extracellular space.

The distribution volume of extracellular markers, e.g. inulin, is believed to be the most valid expression of the size of the extracellular space (Katzman & Pappius 1973) but the method requires a long equilibration time and subsequent tissue analysis.

The method here presented is based on continuous determination of the extracellular concentration of extracellular markers and is therefore able to detect rapid changes of brain extracellular space volume.

## MATERIALS AND METHODS

In brief, the method was applied in the following way. After removal of the dura, the exposed parietal cortex was irrigated with isotonic mock CSF containing 50 mM choline chloride or 50 mM trimethyltin(hydroxymethyl)trimethyl ammonium chloride (N-TRISCl). The extracellular concentration of these substances in the brain cortex was measured by means of microelectrodes during the course of CSD or brain ischemia. Since both substances have low cellular permeability, rapid changes of the concentration reflect changes of the size of the extracellular space.

### Theory

Upon application to the surface of the brain, choline or N-TRIS diffuses into the cerebral cortex through the



Alumina powder as given when necessary. A glass tube is placed through tracheostomy and polyethylene tubes are inserted into the femoral artery and vein for recording of blood pressure and for drug administration. The body temperature was kept at 37°C by a servo-controlled heating system. The animal was breathing spontaneously throughout the experiment. The level as secured in headholder the calvaria was exposed and a hole (about 6 mm in diameter) drilled in the left parietal bone. A smaller hole was made in the left frontal bone about 5 mm in front of the large hole. A perspex ring, 7 mm in inner diameter and 5 mm thick was glued to the bone of the large hole using Eastman 988 adhesive. The dura, as removed, care being taken not to damage the underlying pia. The EEG was recorded with platinum pins inserted beneath the pericranium in the vicinity of the larger hole. In three animals, a decerebrogram was recorded from a small silver disc placed lightly on the parietal cortex with no interference with the local blood supply. The animal was decapitated grossly with glass tube filled with 150 mmol/l sodium chloride in agar placed in neck muscle or in the suboccipital tissue on the right side of the head.

#### Fluoride and solutions

Double-barrelled potassium-selective microelectrodes with tip diameter of about 1 µm were constructed and used as described by Hansen (1977). The electrodes were filled with potassium ion exchanger (Corning 477317). The exchanger has previously been shown to be sensitive to quaternary ammonium ions (Nieber & Lux 1973; Böhm & Simon 1976; Scholer & Simon 1977).

The parietal cortex was irrigated with shock CSF containing, in mmol/l: CholineCl (ChCl) 50, NaCl 75, KCl 15, CaCl<sub>2</sub> 1.3, MgCl<sub>2</sub> 1.1, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO<sub>4</sub> 0.5, urea 2, and Glucose 3.4. ChCl replaced 90 mmol/l of NaCl. The pH is adjusted to 7.4 and the osmolarity was 295 mmol/l. In three animals, ChCl was replaced by 90 mmol/l of trimethylsilyldimethylsilyltrimethylammonium chloride (N-TRISCl), which is ordinary Tris since the hydrogen atoms in the amino-group have been substituted by methyl groups. N-TRIS possesses more hydrophobic groups (CH<sub>3</sub>OH), presumably making the molecule less permeable in the cell membrane. The electrodes were calibrated with 38°C artificial CSF containing ChCl in the following concentrations (mmol/l): 1.5, 5, 10, 20, 40, 60 and with solutions with 10 and 100 mmol/l KCl. For calibration of N-TRISCl the concentrations in the artificial CSF was 50, 25, 1.5, 6.25, 10 (mmol/l). The increase of ChCl, N-TRISCl and KCl is balanced by corresponding decrease of NaCl to preserve isotonicity.

The selectivity constants of choline and N-TRIS with respect to potassium are determined from an extended Nernst equation

$$E = E_0 + \frac{RT}{zF} \ln \left( C_0 + \frac{K_0}{z} [K]^{\frac{z_0}{z}} \right) \quad (6)$$

where  $E$  is the electrode potential,  $E_0$  constant reference potential,  $C_0$  the concentration of choline or N-TRIS,  $K_0$  the selectivity constant,  $z$  and  $z_0$  the valences of choline or N-TRIS and potassium, respectively and  $RT/F$  the Nernst factor.

#### Trimethylsilyldimethylsilyltrimethylammonium chloride (N-TRISCl)

To a stirred mixture of tri(hydroxy)methylmethylamine (0.244 g) sodium hydrogen carbonate (1.0 g) and dimethyl sulfoxide (3 ml) was added methyl iodide (0.7 ml) in the course of 30 min. Stirring at room temperature was continued for 22 h. After dilution with methanol, the solid material was filtered off. Water was added to the filtrate and the solution passed through an ion exchange column (Amberlite IR 120, 70 cm, H-form). After thorough washing with water, the product was eluted with 1M HCl. After evaporation to dryness on rotary evaporator the material was extracted with hot ethanol. Filtering, evaporation and recrystallization from ethanol gave 0.326 g (81%) of almost pure compound (NMR). The compound was recrystallized once more using ethanol-2-propanol as solvent, before being used for the physiological experiments.

Mp 241°C (uncorrected gas evolution). <sup>1</sup>H NMR spectra were recorded in D<sub>2</sub>O on JEOL C-60 HL instrument, using 3,3-tetradecateno-3-(trimethylsilyl)-propionate as internal standard. NMR data: δ 3.35 (9H singlet), 4.06 (6H slightly broadened singlet), 4.7 (singlet) (Found: C 41.84, H 9.04, N 6.96. Calc. for C<sub>14</sub>H<sub>27</sub>NO<sub>2</sub>Cl: C 42.11, H 9.09, N 7.01).

#### Experimental procedure

In order to increase brain extracellular concentration of choline or N-TRIS, the parietal cortex was exposed to warm (38°C) CSF containing 90 mmol/l of the particular test substance. The perspex ring around the large hole functioned as a reservoir and the fluid in it was renewed at least every 15 min.

In three experiments, the ion-selective electrode was positioned at selected depths in the cortex before superfusion with the choline or N-TRIS-CSF in order to follow the initial rate of concentration. In other animals,

lumbar determinations of the concentration were made at different times after onset of irrigation. The CSF was soaked up and the electrode quickly lowered into the cortex by means of mechanically driven micromanipulator. The level at which the electrode made contact with the cortex was considered zero depth. CSF was then added to the reservoir. The electrode was lowered into the cortex and readings of the concentration taken every 100 µm down to a depth of 1000 µm.

In order to assess the distribution of choline or N-TRIS between intra- and extracellular space, extracellular concentration profiles were obtained after 0.5, 2, and 4 h exposure, during which period CSD or ischemia were not elicited. Then the surface was quickly flushed with normal CSF, the rats decapitated, and a 0.75 mm thick slice of cortex cut off with a string knife (Franck et al. 1964).

A sample of the cortical slice from the irrigated part was homogenized by ultrasound with water added. The choline and N-TRIS concentration in the sample was determined with microelectrode calibrated with similarly homogenized control brain samples with known amounts of choline or N-TRIS added. The concentrations in brain water were calculated assuming water content in brain cortex of 80%. From the measured concentration profiles, mean concentration of choline or N-TRIS in the

extracellular space. Assuming diffusion in one dimension and ignoring carrier mediated transport, the change of the extracellular concentration can be treated mathematically by a model concept as shown below.

Consider a layer of cortical tissue, situated at depth  $x$ , oriented parallel to the brain surface (i.e. perpendicular to the direction of the diffusional transport) and having an area of  $1 \text{ cm}^2$  and a thickness of  $dx$ . In this tissue element of volume  $dx \text{ cm}^3$  and with an extracellular concentration of tracer substance  $C_e = C_e(x, t)$ , the change in content of tracer substance within time  $dt$  in its extracellular space can be expressed by a term for diffusive net entrance and a term for loss by penetration through cell membranes (i.e. brain cell membranes and capillary walls). Assuming the intracellular and blood concentration to be zero, the equation becomes

$$dC_e \cdot dx \cdot f_e = [J(x) - J(x+dx)] \cdot dt - \bar{P}_{app} \cdot S \cdot dx \cdot C_e \cdot dt \quad (1)$$

where  $J$  is diffusive flux (per  $\text{cm}^2$  of brain area),  $f_e$  is fractional volume of extracellular fluid in cortical tissue and  $S$  is membrane surface area (per  $\text{cm}^2$ ) of cortical tissue.  $\bar{P}_{app}$  is an apparent weighted mean membrane permeability, the magnitude of which depends partly on the permeabilities of the membranes through which penetration occurs and partly on electrical potentials existing across the membranes. It is therefore a weighted mean of the apparent permeabilities of the various categories of membranes, each of which again is the actual permeability multiplied by a factor determined by the transmembrane potential. Assuming that penetration occurred exclusively into cells with identical intracellular potential ( $V$ ) and that the penetration of the monovalent cations in question occurs only by electrodiffusion, the relation between  $\bar{P}_{app}$  and the actual permeability  $P$  can be calculated from the Goldman flux equation

$$\bar{P}_{app} = P \frac{VF}{RT} \frac{\exp(-(VF)/(RT))}{1 - \exp(-(VF)/(RT))}$$

where  $F$ ,  $R$ , and  $T$  have their usual meaning. For  $V = -60 \text{ mV}$ , the result is  $\bar{P}_{app} = P \cdot 2.5$ , i.e. a uniformly existing transmembrane potential of this magnitude would cause an apparent increase in the permeability by a factor of 2.5. Rearranging eq. (1)

$$\frac{\partial C_e}{\partial t} = \frac{\partial J}{\partial x} - \frac{\bar{P}_{app} \cdot S}{f_e} \cdot C_e \quad (2)$$

and inserting,

$$J(x) = -D \cdot f_e \cdot \frac{\partial C_e}{\partial x} \text{ and } \frac{\bar{P}_{app} \cdot S}{f_e} = \frac{\bar{P}_{app} \cdot S}{f_e} \cdot \frac{C_e}{C_e} = \frac{1}{\tau}$$

where  $1/\tau$  is a rate constant for penetration through the cell membranes (i.e. the fraction of the marker contained in the extracellular volume which leaves it by penetration per unit time), the governing equation is obtained

$$\frac{\partial C_e}{\partial t} = D \cdot \frac{\partial^2 C_e}{\partial x^2} - \frac{C_e}{\tau} \quad (3)$$

which describes diffusion with superimposed penetration into an infinitely large sink.

Eq. (3) assumes unidirectional flux into cells & capillaries. The treatment thus disregards a possible decrease of the net flux with time caused by an increase in the intracellular volume or a fraction of it, if concentration of marker is built up in these volumes. The influx. Since choline and N-TRIS are positively charged, their tendency to leave cells is low compared to the influx, as long as the membrane potential is below the order of  $-40$ – $80 \text{ mV}$  and intracellular concentration below extracellular concentration. Since choline & N-TRIS are both quaternary ammonium compounds, all three N-linked hydrogens substituted by methyl groups, they will be positively charged at all physiological pH values.

The increase of the extracellular concentration in the cortex following the onset of irrigation can be solved under the boundary conditions

$$C_e(0, t) = C_0$$

$$C_e(x, 0) = 0$$

by Laplace transformation. The result is,

$$C_e(x, t) = C_0 \left\{ \exp\left(-\frac{x}{L}\right) \operatorname{erfc}\left(\sqrt{\frac{x}{2D\tau}} + \frac{1}{\tau}\right) + \exp\left(\frac{x}{L}\right) \operatorname{erfc}\left(\sqrt{\frac{x}{2D\tau}} + \frac{1}{\tau}\right) \right\}$$

where  $L = \sqrt{D\tau}$  is the characteristic diffusion length in extracellular space. When the extracellular concentration of tracer substance is constant with time, i.e.

$$\frac{\partial C_e}{\partial t} = 0 \text{ eq. (3) reduces to } D \frac{\partial^2 C_e}{\partial x^2} = \frac{C_e}{\tau}$$

the only physically meaningful solution of which is,

$$C_e(x, \infty) = C_e(0, \infty) \exp\left(-\frac{x}{L}\right)$$

Thus the concentration in the cortex in the steady state decreases exponentially with distance and therefore yields a straight line in a semi-logarithmic plot.

#### Determination of $D$ and $\tau$

The time course of the concentration was measured at different depths in the cortex following irrigation of surface. From the concentration versus time curves obtained at various depths  $D$  and  $\tau$  were found, namely by insertion of different sets of  $D$  &  $\tau$  values into eq. (4).

In the steady state, concentrations in the cortex were plotted versus depth (up to  $1 \text{ mm}$ ) on a logarithmic scale and  $L = \sqrt{D\tau}$  determined as the abscissa value corresponding to a concentration of  $1/e$  of the concentration at the surface.

#### Preparation of animals

Male Wistar rats weighing 400–500 g were used. They were initially anaesthetized with chlorbutol (50 mg/kg)

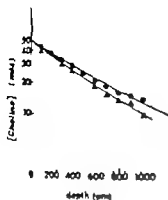


Fig. 3 Extracellular choline concentration (logarithmic scale) in brain cortex following irrigation of brain with isotonic mock CSF containing 50 mM of cholineCl for 2 h ( $\Delta$ ) and 4 h ( $\bullet$ ). The symbols represent mean values from 3 rats.

#### Extracellular concentration of choline and N-TRIS versus time

Irrigation of the parietal cortex with artificial CSF containing 50 mmol/l choline or 50 mmol/l N-TRIS neither interfered with the electrical activity of the cortex nor affected the appearance of foci.

Fig. 3 shows the rise of extracellular choline concentration at two depths in the cortex following onset of irrigation. The dotted lines represent the experimental readings, the unbroken lines the best fit using eq. (4) with the following parameters: At 20  $\mu$ m,  $D=1 \times 10^{-6}$  cm<sup>2</sup> s and  $\tau=25$  min. At 400  $\mu$ m,  $D=2 \times 10^{-6}$  cm<sup>2</sup> s and  $\tau=3$  min.  $D$  for choline in saline is  $14 \times 10^{-6}$  cm<sup>2</sup> s<sup>-1</sup> (Frömster 1970). A similar experiment with the electrode placed at a depth of 400  $\mu$ m, using N-TRIS gave  $D=1.5 \times 10^{-6}$  cm<sup>2</sup> s and  $\tau=60$  min.

#### Extracellular concentration of choline and N-TRIS versus depth

Fig. 3 shows the extracellular choline concentration, measured every 100  $\mu$ m during electrode penetration into the parietal cortex, after 2 and 4 h exposure with 50 mM choline-CSF. CSD or ischemia was not elicited prior to the determination of the depth profile. The values were plotted semilogarithmically and it is likely that the profiles at 2 and 4 h are sufficiently close to indicate that the extracellular content of tracer substance was constant after 4 h. It is, however, not a steady state

since in that situation the intracellular concentration of tracer substance must exceed the extracellular concentration by a factor of ten (see below) assuming an intracellular potential of -60 mV. From the 4 h values in Fig. 3  $L$ , the characteristic length (eq. (5)) was determined to be 740  $\mu$ m. As  $L=1/\sqrt{D\tau}$  and assuming  $\tau=3$  min this gives  $D=4 \times 10^{-6}$  s.

#### Intracellular concentration of choline and N-TRIS

Fig. 4 shows the calculated mean intracellular concentration of choline and N-TRIS in the outermost 0.75 mm of cortex following various times of irrigation. For both compounds, there is a linear concentration rise with time. After 4 h the mean extracellular concentration of choline in two rats was 31 mM and 29 mM while the concentration of choline in the water phase of the homogenized brain was 33 mM and 29 mM respectively; thus at this time nearly equal mean concentrations should be present in the intra- and extracellular compartment. In the rat irrigated with N-TRIS-CSF for 4 h, the mean concentrations in extracellular water and in brain water were found to be 31 mM and 4 mM respectively.

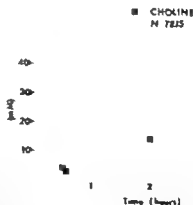


Fig. 4 Mean intracellular concentration of choline or trimethyltris(hydroxymethyl)aminomium ion (N-TRIS) ( $\blacksquare$ ) in the outermost 0.75 mm of rat parietal cortex, irrigated with isotonic mock CSF containing 50 mM of cholineCl ( $\blacksquare$ ) or 50 mM of N-TRISCl ( $\blacktriangle$ ) for 0.5, 2, and 4 h. The intracellular concentration was determined from the total cortical concentration and the extracellular concentration, assuming an extracellular space of 20%.

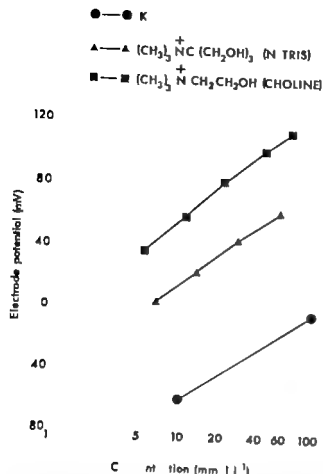


Fig. 1 Calibration curves for an ion selective micro-electrode with  $K^+$ -ion exchanger (Corning 417377) against  $KCl$  (●—●), trimethyltris(hydroxymethyl)methyl ammoniumCl (▲—▲) and cholineCl (■—■).  $NaCl$  was added to the potassium solutions to preserve osmolality. The two latter compounds were dissolved in mock CSF and replaced equimolar amounts of  $NaCl$ .

extracellular space of the outermost 0.75 mm of cortex was determined. From this concentration and a assumption that it amounts to 20% of brain cortex volume, a mean concentration in the corresponding intracellular water was calculated.

With the electrode positioned at depths ranging from 200–700  $\mu m$ , CSD's were elicited from the cortex beneath the small hole in the frontal bone by a drop of saturated  $KCl$ -solution or by quickly pinning a needle into the cortex. CSD was monitored by the transient change of the brain extracellular potential ( $V$ -potential) so-called DC-potential (Leão 1947).

Cerebral ischemia was induced either reversibly by inflation of a pneumatic cuff placed around the neck (Siemkiewicz & Hansen 1978) or irreversibly by arresting the heart by infusion of 0.3 ml saturated  $MgCl_2$ -solution through the venous catheter. In order to avoid artefacts from movements of the brain during early ischemia, the electrode was in some cases removed and quickly repositioned after the cuff had been inflated.

Results are expressed as mean  $\pm$  S.E.

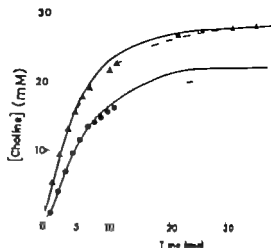


Fig. 2 The rise of choline concentration in the extracellular space in rat brain cortex following impalement of parietal cortical surface with mock CSF in which  $NaCl$  was replaced by 50 mM cholineCl. The choline concentration was measured by an ion-selective micro-electrode at a depth of 700  $\mu m$  (▲) and 400  $\mu m$  (●). Unbroken lines represent best fits to the experimental data using a particular combination of diffusion coefficient for choline and time constant  $\tau$  (see Methods).

## RESULTS

### Electrode sensitivity to choline and N TRIS

The mixed solution technique yielded a  $K_{choline} = -7.2$  indicating the electrode to be about 150 times more sensitive to choline than  $K^+$  in agreement with the finding of Scholzer & Simon (1972). Similarly  $\log K_{NTRIS}$  was 1.1. Since the extracellular  $K^+$  concentration rises to 60 mM above 60 mM during CSD and ischemia (Hansen 1977, 1979; Vyskočil et al. 1977) the choline-induced potentials were measured in solutions of 4 mM  $ChCl$  in 60 mM  $KCl$  or 60 mM  $NaCl$ . Likewise N TRIS-induced potentials were measured in solutions of 25 mM N TRIS with either 60 mM  $KCl$  or 60 mM  $NaCl$ . No differences were detected among the  $K^+$  and  $Na^+$ -containing solutions.

Fig. 1 shows calibration curves obtained with pure solutions of  $ChCl$ , N TRISCl and  $KCl$ . For choline there was an increase in potential of approximately 66 mV for a 10-fold change in concentration above 70 mmol/l and 73 mV below 70 mmol/l. For N TRIS there was a potential change of approximately 65 mV for a 10-fold change in the whole range. The reason for this "super Nernstian" slope is presently unknown.

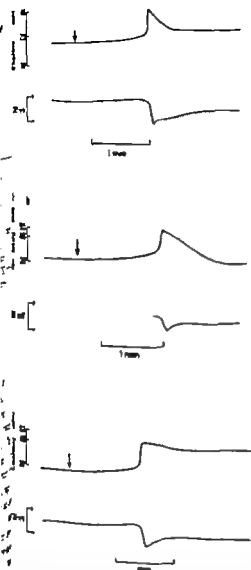


Fig. 7. Change of extracellular perineal cortical concentrations of the brain extracellular space markers during heart arrest. Across indicates heart arrest produced by rapid injection of 0.3 ml saturated  $MgCl_2$ -solution. The traces are irrigated with isotonic Ringer solution containing either 0.3 M cholineCl (a) or 50 mM trimethylammonium ion (N-TRIS) (b), and the concentration of these markers measured approximately 0.5 mm below cortical surface by double-barrelled microelectrodes. Lower curves of each figure represent changes of brain extracellular potential. The duration of irrigation was 1 h in (c) and (d), and 4 h in (e) and (f). Note the decline of marker concentration following the steep increase after irrigation of 1 h, while after 4 h the concentration remained at a high level.

shot should decrease with duration of exposure to marker substances. Since the brain cells are depolarized during CSD (Higashida *et al.* 1974; Sugaya *et al.* 1975) the driving force for transport into the cells is diminished when the concentrations in the intra- and extracellular spaces have become equal (at about 4 h for choline, cf. above). When CSD was elicited after longer irrigation periods, the early decline disappeared (Fig. 5b) and the undershoot was diminished. Fig. 6 shows that the undershoot—relative to the pre-existing concentration—after the first CSD was related to the length of exposure to the marker substance. When ~3 CSD's were elicited repetitively the pre-CSD level of tracer substance dropped for every CSD and the undershoot was progressively diminished. After these CSD's, the concentration of tracer substance returned slowly to a level similar to that before the elicitation of the CSD's due to diffusion from the cortical surface. Thus, when several CSD's were elicited, irrespective of duration of irrigation, the undershoot of the last CSD was similar to the undershoot of the first CSD elicited after 4 h of irrigation. This observation indicates that CSD promoted uptake of tracer substance into cells.

We can make a rough estimate of the extent to which cellular uptake influences the maximal level attained during the steep tracer increase by assuming the time constant for cellular uptake to be similar to the time constant for the concentration decline observed immediately following the end of the steep increase. The amount of choline  $M$  taken up by the cells of  $1 \text{ cm}^2$  of cortex equals  $P_{\text{app}} S C_e (t_2 - t_1) / C_e (t_2 - t_1)$ . In Fig. 5 the choline concentration doubled in  $t_1$  during the steep increase, and  $t_2$  for the early decline was 10 s. During the 2 s, the extracellular choline concentration and the fractional volume of extracellular fluid changes from  $[C_e(1), V_e(1)]$  to  $[C_e(2), V_e(2)]$ . Assuming that the increase of intracellular choline concentration is negligible and that  $t_1$  and  $C_e$  change linearly  $M = [C_e(1) + C_e(2)] \cdot 0.0693 \cdot \frac{1}{2} (C_e(1) + C_e(2)) \cdot t_1$ . The amount of choline taken up by the cells can also be expressed as  $M = [C_e(1) - C_e(2)] \cdot V_e(1)$ . Since  $C_e(1) = 22 \text{ mM}$  and  $C_e(2) = 42 \text{ mM}$  (Fig. 5a) the fractional change of extracellular space volume calculated as  $C_e(1)/C_e(2)$  is  $V_e(2)/V_e(1) = 0.52$ , whereas when cellular uptake is taken into account, the calculation yields  $V_e(2)/V_e(1) = 0.45$ . Thus, the fractional change in extracellular volume, calculated from the fractional change in the choline concentra-



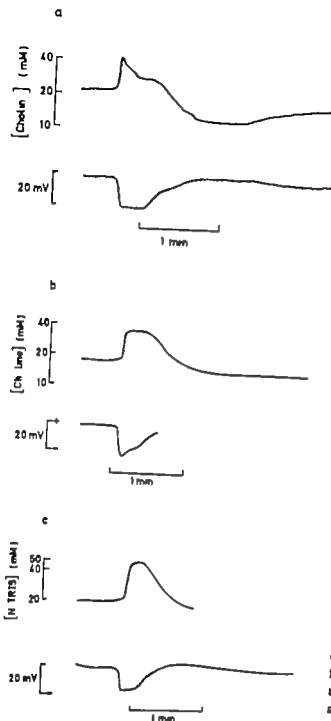


Fig. 5 Change of parietal cortical extracellular concentration of the two extracellular space markers during the course of spreading depression. The cortex was irrigated with isotonic mock CSF containing 50 mM of cholineCl for 1.3 h (a) or 4 h (b). Note greater undershoot at 1.3 h. In (c) the cortex was irrigated with mock CSF containing 50 mM of trimethyltris(hydroxymethyl)methyl ammonium ion (N-TRIS) for 1.6 h. Concentration of the markers was measured approximately 0.5 mm below cortical surface. Lower curve of each figure represents change of brain extracellular potential. Spreading depression was elicited from frontal cortex either by placing a drop of saturated KCl-solution or by a stab with a small needle.

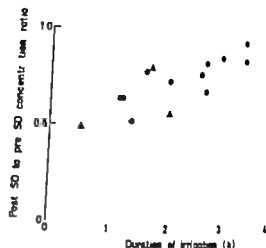


Fig. 6 Relationship between duration of irrigation parietal cortex with isotonic mock CSF containing 50 mM of cholineCl (●) or 50 mM of trimethyltris(hydroxymethyl)methyl ammonium chloride (N-TRIS) (▲) a maximal undershoot of the extracellular concentrations of these substances after the first CSD expressed as ratio of pre-CSD level. Spreading depression was elicited as described in Fig. 5.

#### Changes during cortical spreading depression (CSD)

The changes of concentration of choline (17 rats) and N-TRIS (3 rats) during CSD were similar and will be described together. Fig. 5a shows the change of extracellular choline concentration during CSD after 1.3 h of irrigation. The change of V potential resembled the change observed in rat with normal composition of the brain extracellular space. Coinciding with the rapid negative deflection of the V potential a steep elevation of the choline concentration occurred in 2 s immediately followed by a decline which after about 1 min undershot the pre-CSD level. Fig. 5b shows CSD in a rat after choline exposure for 4 h. Note that it shows a similarly steep increase of the choline concentration as in Fig. 5a but the increase is followed by a plateau phase and there is less undershoot. Fig. 5c shows the change of the N-TRIS concentration during a CSD after 1.6 h of irrigation. A similar concentration change as that of Fig. 5a was found.

The early decline of choline concentration immediately after the steep increase (Fig. 5a) and the undershoot after CSD could reflect expansion of the extracellular space or removal of marker substance from it. If cellular uptake was involved the decline should occur at a slower rate and the under-

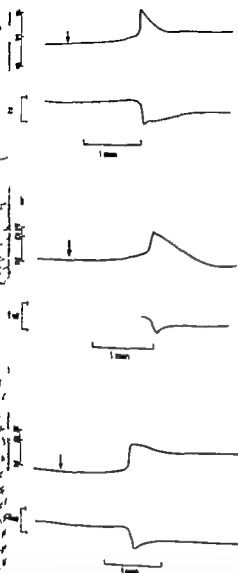


Fig. 7. Change of extracellular peritumoral cortical concentrations of the two extracellular space markers during CSD. Arrows indicate heart arrest produced by rapid suction of 0.3 ml saturated  $MgCl_2$ -solution. The cortex was irrigated with isotonic mock CSF containing either 50 mM cholineCl (a, c) or 50 mM trimethylsilylacetamide ion (N-TRIS) (b, d), and the concentration of these markers measured approximately 0.5 mm below cortical surface by double barrelled microelectrodes. Lower curve of each figure represents change of brain extracellular potential. The duration of irrigation was 1 h in (c) and (d), and 4 h in (a, b). Note the decline of marker concentration following the steep increase after irrigation of 1 h, while after 4 h the concentration returns

shot should decrease with duration of exposure to marker substances. Since the brain cells are depolarized during CSD (Higashida et al 1974, Sugaya et al 1975) the driving force for transport into the cells is diminished when the concentrations in the intra- and extracellular spaces have become equal (at about 4 h for choline cf. above). When CSD was elicited after longer irrigation periods, the early decline disappeared (Fig. 5b) and the undershoot was diminished. Fig. 6 shows that the undershoot—relative to the pre-existing concentration—after the first CSD was related to the length of exposure to the marker substance. When 2–3 CSD's were elicited repetitively the pre-CSD level of tracer substance dropped for every CSD and the undershoot was progressively diminished. After these CSD's the concentration of tracer substance returned slowly to a level similar to that before the elicitation of the CSD's due to diffusion from the cortical surface. Thus, when several CSD's were elicited irrespective of duration of irrigation the undershoot of the last CSD was similar to the undershoot of the first CSD elicited after 4 h of irrigation. This observation indicates that CSD promoted uptake of tracer substance into cells.

We can make a rough estimate of the extent to which cellular uptake influences the maximal level attained during the steep tracer increase by assuming the time constant for cellular uptake to be similar to the time constant for the concentration decline observed immediately following the end of the steep increase. The amount of choline  $M$  taken up by the cells of  $1 \text{ cm}^2$  of cortex equals  $P_{\text{max}} S C_0 t = f_0 / t_f C_0 t$ . In Fig. 5a the choline concentration doubled in 2 s during the steep increase and  $t_f$  for the early decline was 10 s. During the 2 s, the extracellular choline concentration and the fractional volume of extracellular fluid changes from  $[C_e(1) f_e(1)]$  to  $[C_e(2) f_e(2)]$ . Assuming that the increase of intracellular choline concentration is negligible and that  $f_e$  and  $C_e$  change linearly  $M = \frac{1}{2} (f_e(1) + f_e(2)) \cdot 0.0693 \text{ s} \cdot \frac{1}{2} (C_e(1) + C_e(2)) \cdot 2 \text{ s}$ . The amount of choline taken up by the cells can also be expressed as  $M = f_e(1) C_e(1) - f_e(2) C_e(2)$ . Since  $C_e(1) = 22 \text{ mM}$  and  $C_e(2) = 42 \text{ mM}$  (Fig. 5) the fractional change of extracellular space volume calculated as  $C_e(1)/C_e(2)$  is  $f_e(2)/f_e(1) = 0.52$ , where as when cellular uptake is taken into account, the calculation yields  $f_e(2)/f_e(1) = 0.83$ . Thus, the fractional change in extracellular volume, calculated from the fractional change in the choline concentra-

tion may be 15% too low. The  $t_1$  for the early decline observed in other CSDs was larger than 10 s and accordingly no correction was performed.

The change of the choline electrode potential during the steep increase of the first spreading depression in 11 rats averaged  $19 \pm 0.9$  mV while the change for all later CSDs averaged  $22 \pm 0.7$  mV ( $n=23$ ) corresponding to an increase from 17 mM to 33 mM and from 17 mM to 35 mM respectively (i.e. increases by 94% and 105%). In all rats the duration of the steep increase was 1–2 s. In ten rats where more than one CSD (2–7) was elicited the undershoot after the last CSD was  $5 \pm 1.1$  mV corresponding to a choline concentration of 85% of the pre-CSD level. The five CSDs in three N-TRIS-treated rats caused an average electrode potential change of  $1 \pm 3$  mV during the steep increase corresponding to a concentration increase from 23 mM to 47 mM (i.e. increases by 104%). The undershoot after the second CSD in two of the rats was 5 mV (81% of pre-CSD level).

#### Changes during ischemia

No apparent differences were noted between the results obtained with choline and those obtained with N-TRIS. Fig. 7 shows the change of the extracellular marker concentration and V potential during brain ischemia. The change of the V potential is similar to the change observed in experiments where the extracellular space contained no foreign ions (Hansen 1977; Vyskočil et al. 1972). Immediately following the heart arrest induced by  $MgCl_2$ -injection the choline concentration slowly began to increase. At the steep negative deflection of the V potential (anoxic depolarization) a 2-fold elevation of choline concentration occurred rapidly. This pattern was observed in all ischemic episodes but the concentration attained after the steep increase depended on the duration of exposure to marker substance. If the surface of the brain was irrigated for approximately 1 h before ischemia, the concentration declined after the steep increase (Fig. 7a,b). After an exposure time of 4 h, however, the level remained almost stable after the steep increase (Fig. 7c).

The fall of marker concentration after the anoxic depolarization could be due to expansion of the extracellular space or more likely to cellular uptake since the concentration fall was more pronounced after short time exposure, i.e. when the

Intracellular marker concentration was low (Fig. 7). The duration of the steep increase was 1–3 s (a  $t_1$  of 1–3 s) while the  $t_1$  for the initial post-potential uptake was above 15 s in all later episodes. Because of the short duration of the steep increase the cellular uptake of choline or N-TRIS may be considered to have had a negligible effect on their extracellular concentrations during the steep increase (cf. section on Changes during spreading depression (CSD)).

The increase of choline potential during the depolarization phase, i.e. before the steep increase, was  $6 \pm 1.0$  mV corresponding to an increase from 16 mM to 20 mM and during the steep increase  $17 \pm 1.1$  mV (from 20 mM to 35 mM) (i.e. an increase to a level 25% and 119% above the ischemic level respectively). In the three N-TRIS-treated rats the values were  $6 \pm 1.1$  mV (from 22 to 27 mM) for the prepolarization phase,  $17 \pm 1.2$  mV (from 27 mM to 45 mM) for the steep increase (23 and 105% above the pre-ischemic level respectively).

In a few instances the change of the choline concentration was studied during and after ischemia induced by strangulation with a pneumatic cuff, a duration of 5–10 min. The increase of choline concentration during ischemia was reversible, the fastest rate of decline being observed during normalization of the V potential. This finding agrees with observations of the normalization of extracellular potassium concentration after a period of ischemia (Hansen et al. 1979). In association with brain swelling noted in the microscope the post-ischemic choline concentration undershot control level, presumably by forcing the tip of the electrode into deeper structures with lower choline concentrations. No brain movements were observed during the course of irreversible ischemia nor during CSD.

#### DISCUSSION

The present investigation aimed to measure the change of extracellular space of brain cortex during CSD and ischemia. Under the assumption that the concentration change of choline and N-TRIS reflects volume changes in the extracellular water phase, the size of the extracellular space decreased to 50% during CSD. Under the same assumption the space shrank during

to 80% of the

value during the depolarization phase and  
-a V during the anoxic depolarization.

#### Choline and N-TRIS as extracellular markers

Choline is chosen as a marker for the extracellular space for three reasons. First, it is generally accepted that cell membranes are not or only very slowly permeated by choline. The compound is often used in studies in which physiologically important ions such as potassium or sodium must be excluded by an inert cation. Secondly, choline permeability of the nerve cell membrane remains low in situations in which the ionic permeability is increased, e.g. during the action potential change (Hille 1971; Hodgkin & Katz, 1948). Thirdly, choline can be accurately measured by means of primary potassium-sensitive microelectrodes without significant interference from potassium (Katz & Oles, 1966). N-TRIS can be measured with the same technique and was selected because it is likely to be less permeable in cell membranes (cf. Bethe 1964).

It is unlikely that the change of marker concentrations observed during CSD and ischemia is not influenced by transport of markers to or away from the extracellular space, since even in the normal state considerable cellular uptake was observed. The cellular uptake of choline in our experiment occurs almost exclusively by electrodiffusion, since facilitated diffusion would only increase the intracellular concentration from approx. 30  $\mu$ M (Günzel 1976) to 3 mM in 4 h, assuming a maximal transport capacity of 10 nmol/g min (Carroll & Goldberg 1975). But the substances are still useful as extracellular markers, if it can be shown that the free constant, for transport from the extracellular space including transport through capillary walls (Cornford et al. 1978) is much higher than for the change of the extracellular space volume. In the normal state, for transport from the extracellular space as above 25 min. However the rate of transport from the extracellular space during CSD and ischemia is unknown but can be inferred from the change of marker concentration occurring immediately after the steep increase. By comparing the change in marker concentration during the steep increase with the change in marker concentration immediately after the end of the steep increase, it was shown that loss of marker substances from the extracellular phase affected the

calculation of the volume changes during the steep increase only negligibly. The presence of this significant albeit slow change of marker concentration following the steep increase indicates an increase of brain cell permeability to the present tracer substances. It invalidates any interpretation of choline and N-TRIS concentrations in terms of extracellular volume for the entire period of time following and possibly prior to the steep increase and it further suggests that the steep increase of marker concentration itself could not be the result of cellular release.

#### Extracellular changes during CSD and ischemia

Maintenance of cellular volume is commonly described by the double Donnan system (MacKnight & Leaf 1977) according to which the effect of the osmotic pressure of the intracellular impermeable anions is counteracted by extrusion of sodium. When this mechanism fails, cells swell because water follows sodium and chloride into the cells (Kow & Van Harreveld 1972; MacKnight & Leaf 1977; Van Harreveld 1966).

The finding of large decreases of extracellular concentration of sodium and chloride during CSD and ischemia (anoxic depolarization) (Hansen 1979; Kraig & Nicholson 1978; Nicholson & Kraig 1975) is in agreement with the result of the present study. During the pre-depolarization phase in ischemia only small changes of extracellular sodium- and chloride concentration take place but cellular swelling could be due to accumulation of anaerobic metabolites intracellularly. Several other works within recent years support the occurrence of extracellular volume changes during the conditions studied above.

Electrical impedance of brain tissue is considered an index of the volume of the extracellular space (Van Harreveld 1972). During CSD a transient 2-fold increase of impedance was found (Hoffman et al. 1973) and in ischemia, a slight increase was found during the pre-depolarization phase followed by a rapid rise during which the impedance almost doubled (Van Harreveld & Ochs 1956).

Brain extracellular space has been studied electromicroscopically using freeze-substitution in order to preserve the water distribution between the intra- and extracellular spaces (Van Harreveld 1972). During CSD and ischemia, the volume of the

extracellular space decreased and cells swelled (Van Harreveld & Malhotra 1965; Van Harreveld & Khattab 1967). A volume increase of cerebral cortical cells during anoxia measured by light scattering has also been reported (Lipton 1973). Altogether the results referred to above are in good agreement with those obtained by the present technique showing shrinkage of the extracellular space to half its initial value during CSD and ischemia.

The authors wish to thank Ms M. Anker Sørensen for expert technical assistance, O. Christensen Ph.D. for the mathematical treatment, Professor C. Crone, A. Gjedde, M.D., H. Lund Andersen, M.D., and Professor P. Olesen-Larsen for valuable advice and support, and Ms H. Ree for expert typing of the manuscript.

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# Renal blood flow distribution during *E. coli* endotoxin shock in dog

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The effect of endotoxin on renal blood flow distribution was studied in anesthetized dogs. Renal blood flow was measured as hydrogen clearance by platinum electrodes placed in outer and in inner halves of cortex and by electromagnetic flow meter. Intravenous injection of *E. coli* endotoxin 3-5 mg/kg b. wt. promptly reduced arterial blood pressure (AP) and renal blood flow. After transient increase for 45 min AP and renal blood flow declined to about 30% of the control 2-3 h after injection. The reduction in outer cortical blood flow (OCF) was not significantly different from the reduction in inner cortical blood flow (ICF). The hematocrit (Hct) increased from  $40.1 \pm 3.8\%$  to  $54.6 \pm 8\%$  but mean renal vascular resistance did not change. Total plasma protein concentration was not significantly elevated. A marked local flow variability was observed in some periods during the phase of shock with declining AP and total renal blood flow at high Hct. Thus renal blood flow showed phasic changes but the OCF/ICF ratio was not changed during endotoxin shock. Local blood flow instability was observed periodically at high Hct.

**Key words:** Renal blood flow distribution, hydrogen clearance, endotoxin.

Ischemic necrosis of the outer renal cortex is less produced by injection of staphylococcus than rabbits by several investigators (Neisser & Reid 1901, Narvaez 1938, Trueta et al. 1947). Neisser and colleagues postulated that renal blood flow is diverted from "cortical" glomeruli toward medullary glomeruli and thereby caused reduction in urinary output. A selective reduction of renal flow in outer cortex was confirmed by Lawrence et al. (1977) by measuring  $^{86}\text{Kr}$  concentration in frozen kidney pieces from dogs in endotoxin shock and by Neberger & Passmore (1978) recording gas washout by external detection. Similar results were obtained also by Croonenwit & Linde (1978) counting microspheres in septic dog kidneys. They held that washout of the medullary cortical concentration gradient by increased juxtaglomerular blood flow could explain the inappropriate decrease observed in their experiments. However, such redistribution has never been proved in any model by use of  $\text{H}_2$  washout technique (Hedberg et al. (1970) observed a homogene-

ous reduction of cortical perfusion in septic patients by external counting of  $^{86}\text{Kr}$ .

During dehydration shock in dog, Kirkebo & Tysssebotn (1977) observed patchy intermittent ischemia equally distributed in both cortical layers. The flow instability was most frequently encountered at medium reduction of arterial pressure and renal blood flow and always at high Hct. Septic shock is more often characterized by higher cardiac output and renal blood than other types of shock (Schoemaker 1977) although conflicting results have been reported for the dog (Hilshaw et al. 1961-1968). Hct is also known to increase rapidly.

Therefore the aim of the present investigation was to measure renal blood flow distribution by  $\text{H}_2$  washout technique during endotoxin shock and to search for the appearance of patchy intermittent changes in cortical blood flow at high Hct. The random and relatively rare occurrence of sudden changes in local flow in the present work, made it difficult to investigate possible mechanisms explaining this phenomenon.





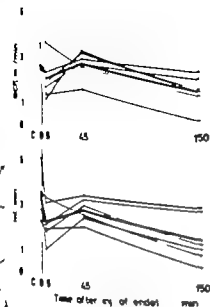


Fig. 1. Outer cortical blood flow (OCF) and inner cortical blood flow (ICF) in control and after injection of *E. coli* endotoxin. 6 dogs. Symbols represent mean local flow in 3 electrodes. Average values given by solid lines.

Fig. 2. The renal arterial blood flow (RAP) averaged  $3.63 \pm 0.77$  ml/min/g in accordance with local outer cortical blood flow (OCF) of  $3.59 \pm 0.85$  ml/min/g and inner cortical blood flow (ICF) of  $1.6 \pm 0.7$  ml/min/g. The  $H_2$  washout curves in several periods were all monoexponential down to at least 18% of initial saturation. TPP was  $4.4 \pm 0.17$  g/100 ml.

#### Effects of endotoxin

Endotoxin administration caused variable haemodynamic responses, but a general pattern may be outlined.

Immediately after the injection of endotoxin AP and RAF dropped to an average of 56% and 4% of the control whereas renal vascular resistance (RR) increased by 35% (Fig. 1). OCF and ICF were reduced to 57% ( $2.03 \pm 0.90$  ml/min/g) and to 67% ( $2.30 \pm 0.84$  ml/min/g) of control about 5 min. after administration (Fig. 1).

The initial phase lasted a few minutes. AP and RAF thereafter increased rapidly for the next 5–10 min, then more slowly. Both parameters reached maxima of about 85% of control 45 min after starting endotoxin injection. In 5 out of 6 dogs RAF transiently increased to or even exceeded the control level (Fig. 1). At 45 min average OCF and ICF were increased to 76% ( $7.74 \pm 0.60$  ml/min/g) and 84% ( $1.90 \pm 0.50$  ml/min/g) of the control. Hct was raised to 50% but RR had returned to the control values after a transient rise (Fig. 1).

From about 45 min after injection AP and RAF fell gradually so that 3 h after start of endotoxin injection average AP and RAF both were close to 50% of control. Although a great scatter in RR was found average RR remained at control level where as mean Hct was increased to  $54.6 \pm 8\%$ . TPP was not significantly different from control. 2 h after injection of endotoxin OCF was reduced to 44% ( $1.56 \pm 0.73$  ml/min/g) and ICF to 50% ( $1.71 \pm 1.04$  ml/min/g). Except for the first 5 min after injection of endotoxin when the OCF/ICF ratio was significantly reduced ( $P < 0.01$ ) the OCF/ICF ratio was not significantly different from control throughout the rest of the experiment.

Generally local blood flow at single electrode sites in each dog was well correlated. However in certain periods between 50 and 120 min after endotoxin injection, a pronounced blood flow heterogeneity occurred in all animals detected as uncorrelated flow variations between single elec-



Fig. 3. Blood flow at single electrode sites in outer cortex (OCF) and in inner cortex (ICF) in control and after injection of *E. coli* endotoxin in one dog, showing local flow instability in periods during the shock with decreasing AP and total blood flow.

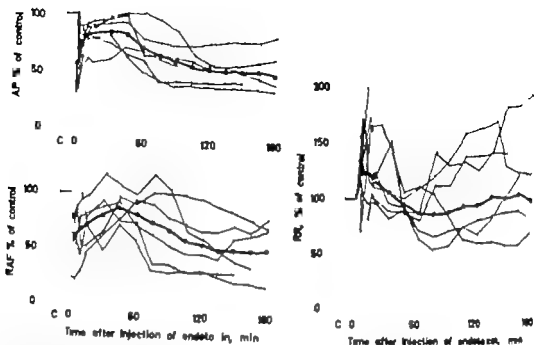


Fig. 1 Renal arterial blood flow (RAF), mean arterial blood pressure (AP) and renal vascular resistance (RR) in control (C) and after injection of *E. coli* endotoxin in 6 dogs. Average values given by solid lines.

## METHODS

The experiments were undertaken on mongrel dogs of both sexes weighing 21–40 kg, mean b.wt. 26 kg. The dogs had free access to water, but food was withheld during the last 16 h before surgery. Anaesthesia was induced by Nembutal i.v. 25 mg/kg b.wt. and adequate anaesthetic level maintained by additional doses. The dogs breathed spontaneously through an endotracheal tube. Polyethylene catheters were placed in a saphenous vein and artery for infusion and blood pressure recording respectively.

The left kidney was retroperitoneally exposed through a flank incision and gently dissected free. A polyvinyl catheter was sewn into the renal artery with the catheter tip directed upstream. An electromagnetic flow probe (Nycotron) of suitable dimension was placed on the renal artery for continuous determination of total renal blood flow. Three L-shaped platinum electrodes with sensitive tapered tips of 0.05–0.15 mm diameter were placed in the outer half and 3 in the inner half of the cortex. The shafts of the electrodes were fixed to the renal capsule with 2 sutures and the electrodes carefully covered with perirenal fat. The wound was closed with towel clips.

The  $H_2$  concentration in the tissue around the electrode tips was determined polarographically at a polarization potential of +0.2 V vs. an Ag/AgCl electrode placed subcutaneously on the hip (Aukland et al. 1973). The electrode current was recorded on a 6-channel recorder (Rikadenki Kogyo Co. Model B-64).  $H_2$  saturated saline at 37°C was injected into the renal artery until a stable current was obtained and then abruptly stopped. The washout curve was recorded and replotted on a semilogarithmic paper. The local blood flow was calculated from

the formula  $f = -\lambda \ln 0.5/T_1$  ml/min/g, where  $T_1$  is half life of  $H_2$  concentration in the tissue around the electrode tips. The tissue/blood partition coefficient ( $\lambda$ ) assumed to be 1.00 (Aukland et al. 1964).

At the end of each experiment the kidney was excised, drained for 3 min and weighed. This weight was used for calculation of total renal blood flow per unit weight of the flow meter reading of total renal blood flow.

Each electrode was excised and the electrode sampling sites carefully examined for hematoma, and the position of the cortex was noted.

The *E. coli* endotoxin (Bacto-Lipopolysaccharide coli 0111:B4, Difco Lab.) was given i.v. in a total dose of 3–5 mg/kg b.wt., first as a bolus injection of about 0.5 mg/kg b.wt. and the rest of the dose within 30 min. 7 experiments were continued for 3 h after start endotoxin injection.

Blood samples were taken approximately each half hr to determine Hct and total plasma protein concentration (TPP) using the amino black method (Munk Plum et al. 1955).

Statistical significance was evaluated by Student's *t*-test for paired data.

## RESULTS

### Control measurements

The control measurements were made 1 h after completed surgery. The average arterial blood pressure (AP) was  $124 \pm 5$  (S.E.) 7.4 mmHg and mean

show that compartment analysis of composite renal curves externally detected disagree with local flow measurements and give unrealistic zonal flow volumes. From tissue sampling technique, interpolation between timed  $^{86}\text{Kr}$  concentrations in different dogs Pastore et al (1977) reported that blood flow in outer cortex was reduced more than in inner cortex and outer medulla 30 min after injection of *E. coli* endotoxin. Their experimental data, however, showed great scatter and especially their control washout curves from each of the zones were still semilogarithmic. The average slope of the regression line used for flow calculation is then strongly depending on the duration of recording. On the other hand, outer cortical volume rate estimated from the initial component could be unreasonably high (ca.  $1 \text{ ml min}^{-1}$ ) compared to total renal blood flow measurements. The low flow components might indicate large localized low flow regions, but this is not supported by measurements with  $\text{H}_2$  electrodes (Tyssebo et al 1979). An alternative and more likely explanation could be redistribution of  $^{86}\text{Kr}$  as suggested by Aukland (1980).

Clausen et al & Lindemann (1978) slowly infused *Pseudomonas aeruginosa* for 90 min in awake dogs. Only a small reduction in AP and a small rise in Hct were observed. Most dogs showed increased total renal blood flow and a redistribution of  $15 \mu\text{m}$  microspheres from outer cortex towards inner cortex, as usually has been found with microspheres during renal vasodilation (Stein et al 1974). Contrary we have recently demonstrated that  $15 \mu\text{m}$  microspheres were redistributed in cortex during acetycholine infusion whereas the distribution of  $10.5 \mu\text{m}$  spheres was not changed as found by  $\text{H}_2$  or antipyrine clearance methods (Tyssebo & Kirkebo 1979; Clausen et al 1979).

#### Local blood flow heterogeneity

In the present experiments a marked variability in local cortical blood flow was found in all dogs in shock during development of the septic shock. It is noteworthy that the present as well as previous findings of intermittent flow changes (Kirkebo & Tyssebo 1977) were detected only at high Hct values, whereas such changes in local flow were not observed during hemorrhagic hypotension with a low Hct. Normal dogs with a normal blood volume compensated for rise in Hct and viscosity by a reduction of peripheral resistance (McDonald 1974;

Seligman et al 1945). When blood viscosity was raised by exchange of packed red cells for whole blood during hemorrhagic shock, then blood flow was further reduced (Seligman et al 1945). The rise in blood viscosity with Hct is likely to be more prominent at states of low flow as during shock, because blood viscosity increases at low rates of shear (Usami et al 1969). Glomerular filtration at high Hct could further rise Hct and viscosity in postglomerular vessels more than at normal Hct. Enhanced aggregation of red cells may also occur at high Hct and during hypoperfusion and has been held responsible for the frequent observations of nonuniform circulation in parallel macrovessels during various disease states (Schmid-Schonbein 1976). At the higher than normal yield stress seen during polycythemia (Reploge & Merrill 1970) reflow after local occlusion may demand a higher pressure head. Rheological factors may thus sustain hypoperfusion but are less likely to initiate arrest of flow.

Delaunay et al (1949) found that endotoxin produced strong waves of contraction along small arteries in guinea pig mesentery and rabbit ear followed by dilatation, indicating that endotoxin may have effect on vascular reactivity. In the present work, patchy intermittent blood flow occurred mainly at moderately lowered AP and renal blood flow. Increasing concentrations of vasoactive agents are measured in such phases of endotoxin shock (Spink et al 1966) possibly also causing labile vasoactivity. On the other hand, RR was maintained due to increased viscosity and not to reduced vessel diameters. High concentrations of vasoactive agents were found also during hemorrhagic hypotension, but patchy intermittent flow changes were never seen (Aukland et al 1973; Aukland 1976). These observations suggest that rheological factors and labile vasoactivity together induce the sudden shifts in local flow.

In conclusion, *E. coli* endotoxin decreased local outer and inner cortical blood flow proportionally. A pronounced local flow heterogeneity was observed in phases of shock when Hct was markedly increased.

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trode sites randomly distributed in cortex. Local blood flow measurements from one experiment are presented in Fig. 3. In 3 dogs sudden sharp changes in washout rate were observed at single electrode sites indicating intermittent and patchy cortical flow distribution without measurable changes in total renal blood flow. The local blood flow could remain at the new value from a few seconds up to 1 h before suddenly returning to or approaching the previous value.

Three years ago a corresponding observation was made (unpublished) on one dog regarded as unsuitable for planned experiments due to migrating pale depressed patches of 0.5–2 cm<sup>2</sup> seen on the renal surface. The sedimentation rate was increased and the blood culture later verified a *Staphylococcus aureus* sepsis. The dog had a normal blood flow (3.2 ml/min/g) but during control measurements sudden abrupt bends were recorded on single washout curves. The occurrence of intermittent patchy flow changes persisted after  $\alpha$  receptor blocking by phentolamine.

## DISCUSSION

The average values for AP and renal blood flow in control period were within normal ranges although slightly higher local blood flow has been obtained previously by electrodes with thin tip diameter (0.05–0.15 mm) as used in the present work (Tyssebotn & Kirkebo 1979).

The administration of endotoxin caused a sharp transitory drop in AP and renal blood flow as observed also by Dedichen & Schenk (1967) and Hinshaw et al. (1968) followed by a shortlasting fairly complete recovery of both AP and RAF. After the immediate transitory rise average RR fell rapidly to control within 30 min and remained low or at control level for the rest of the experiment. Because Het rose markedly in the same period and accordingly also blood viscosity (Usami et al. 1969) a vasodilatation of the renal vessels actually occurred. Since plasma protein concentration did not increase significantly the rise of Het probably was due to leakage of plasma proteins and fluid from the capillaries. Severe sepsis in man often exhibits a hyperdynamic state characterized by a low total peripheral resistance and a high cardiac index (Shoemaker 1971; Wilson et al. 1965) and renal plasma flow (Lucas et al. 1973). On the contrary it has been held that in the dog a fall in cardiac output

and a rise in total peripheral resistance occur in response to endotoxin and that dog experiments therefore represent poor models of the septic patients (Waisbren 1964; Wilson et al. 1965; Lucas et al. 1973). The septic shock of diverse origin in already severely ill patients may certainly develop differently from acute endotoxin shock in healthy dogs but the differences between species may have been exaggerated. Thus Gombotz et al. (1968) demonstrated that intravenous administration of pyrogen in man produced an initial renal vasoconstriction and a subsequent renal vasodilatation as seen in our dog. While Hinshaw et al. (1968) reported that lethal doses of endotoxin resulted in renal vasoconstriction they later found that was unchanged (Hinshaw et al. 1968). Increased renal blood flow was found in dogs receiving lethal doses of endotoxin or *E. coli* cells (Dale et al. 1976). Hermreck & Thal (1969) likewise measured a 60% increase of renal blood flow in dogs with septic hind limbs. Dedichen and Schenk (1967) however found a gradual rise in RR after let doses of endotoxin but concluded that the moderate increase was due mainly to decreased vascular filling pressure and increased viscosity rather than to active vasoconstriction. Taken together it is indicated that the procedure for inducing the endotoxin shock may influence the magnitude and duration of vascular responses but that the change of RR in dog is often moderate. As RR remained control level while Het rose in the present experiments a fall in renal hindrance was evident.

### Cortical blood flow distribution

Local cortical blood showed the same phasic responses to endotoxin as RAF. The reduction of OCF was not significantly greater than the reduction in ICF except in the unstable phase 5 min after endotoxin injection.

By external counting of <sup>125</sup>I Neiberger & Passmore (1978) found that 30 min following endotoxin injection component I and II were found but 2 h later both components were present. Using the same technique with <sup>133</sup>Xe Cortez et al. (1977) did not find any redistribution of intrarenal blood flow in two septic patients. Hollenberg et al. (1977) found that the first rapid component disappeared in patients with renal failure of diverse etiology including nephrotoxic sepsis and shock but it suggested a persistent homogeneous reduction of cortical perfusion. However Aukland (1976) has

# Sodium nitroprusside induced cGMP accumulation in isolated frog skin epithelium. Effect on cAMP, hydroosmotic and natriuretic response to antidiuretic hormone

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JOHNSEN A. H. & NIELSEN R. Sodium nitroprusside induced cGMP accumulation in isolated frog skin epithelium. Effect on cAMP, hydroosmotic and natriuretic response to antidiuretic hormone. *Acta Physiol Scand* 1980 108: 373-379. Received 8 July 1979. ISSN 0001-6772. Institute of Biological Chemistry, August Krogh Institute, Copenhagen, Denmark.

The cGMP content of isolated frog skin epithelium has been measured, and the basal level was found to be  $14.3 \pm 1.7$  fmol/mg dry weight. 0.1 mM sodium nitroprusside induced 10-fold increase in the cGMP level within 5 min after which it rose more slowly. The maximum increase in cGMP level was obtained with 1 mM sodium nitroprusside giving 20-30-fold increase. 1 mM sodium nitroprusside per se had no effect on osmotic water flow or active sodium transport. On the other hand, the osmotic water flow response to arginine vasotocin was somewhat enhanced in skins which had been pretreated with 1 mM sodium nitroprusside; thus the water flow responses to 1 and 31 ng/ml arginine vasotocin were on the average 31 and 14% higher in skins exposed to sodium nitroprusside than in control skins. Sodium nitroprusside had no effect on the increment in sodium transport rate elicited by arginine vasotocin. Sodium nitroprusside alone increased the cAMP level slightly; the enhanced cAMP level reached after 30 min incubation with 40 ng/ml arginine vasotocin, was 20% higher in the presence of 1 mM sodium nitroprusside. In conclusion: cGMP has no effect on osmotic water flow nor on active sodium transport and is not involved in the regulation of sodium transport by antidiuretic hormone. However, cGMP (or sodium nitroprusside) has a moderate effect on the hormone-stimulated osmotic water flow.

It is well established that ADH increases active sodium transport and water permeability in various epithelia. It is also generally accepted that at least some of the effects of ADH are mediated by cAMP (for example Strewler & Orloff 1977). Whether cAMP is the other common cyclic nucleotide involved in salt and water regulation is less clear. Orloff & Handler (1967) have suggested cAMP and cGMP as mediators of the hydroosmotic and natriuretic response to ADH respectively. Bourgoignie et al. (1969) have found that cGMP, in contrast to cAMP, has no effect on OWF but like cAMP stimulates SCC. This finding has not been confirmed by Bentley (1970) who found no effect of cGMP on OWF or SCC, nor by Sugita et al. (1973), who tested several different preparations

of cGMP in different Ringer's solutions. However, Sugita et al. (1973) have found another effect, namely that cGMP enhanced the SCC response to ADH. In 2 out of 3 series Piccini & Parisi (1973) have investigated the combined effect of cGMP and ADH on OWF and found that cGMP increases the basal level a little but strongly inhibits the response to ADH. cAMP and to a combination of theophylline and cAMP.

Abbreviations used in this paper: ADH, antidiuretic hormone; AVT, arginine vasotocin; cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, guanosine 3',5'-cyclic monophosphate; OWF, osmotic water flow; SCC, short circuit current; measure of active sodium transport; SNP, sodium nitroprusside.

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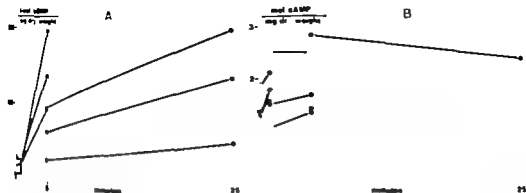


Fig. 2. The effects of varying periods of incubation with  $10^{-4}$  M SNP on concentrations of cGMP (a) and cAMP (b). To compensate for the rather biological variation (especially evident in this figure (a) at 5 and 25 min) the experiments were performed on symmetrical pieces. 3 series of expts. were made by incubating symmetrical pieces for the following periods: 0 and 1 min, 1 and 5 min, 5 and 25 min. The results obtained with symmetrical pieces are connected by lines.

ml for dry weight determined. The supernatant was extracted 4 times by 7 volumes water-saturated diethylether and lyophilized. The lyophilizate was dissolved in 500  $\mu$ l water, placed on a small column (E. cryogen Scientific) with 14 g dry  $Al_2O_3$  and eluted with 10 ml water. The eluate was lyophilized, dissolved in buffer and cGMP measured by radioimmunoassay of the acetyl-derivative (Freeman & Krabben 1976). In an other aliquot cAMP was measured by the method of Getshier et al. (1977). The partition on  $Al_2O_3$  removes substances which interfere with the cAMP assay (Johansen & Naefen 1978). The overall recoveries were typically 55% for cGMP and 75% for cAMP.

The specificity of the cGMP antiserum was tested

against 3'-GMP, 5'-GMP, 5'-GDP, 5'-GTP, 2',3'-cGMP, 2',3'-cAMP, 3',5'-cAMP and 5'-ATP (GTP was obtained from Sigma, the other nucleotides from Boehringer). Only 3'-GMP, 5'-GDP and 3',5'-cAMP interfered detectably in the assay. If the nucleotides are not lost in the standard extraction and purification procedure the lowest detectable cellular concentrations would be approximately  $10^{-8}$ ,  $10^{-4}$  and  $10^{-7}$  M respectively. But of these 3 nucleotides only cAMP is eluted from  $Al_2O_3$  (Wilde 1974) therefore only cAMP could interfere in the assay. The actual cAMP concentrations in the epithelia were less than  $10^{-6}$  M (Fig. 2b, Fig. 3b and Table 3). 1 mg dry weight correspond to 2.3  $\mu$ l cell water, consequently much less than the  $10^{-6}$  M necessary to give interference in the cGMP assay. Thus

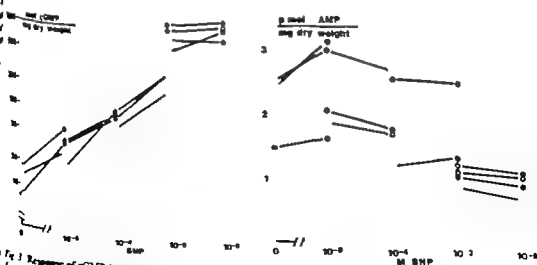


Fig. 3. Response of cGMP (a) and cAMP (b) to 5 min incubations with different concentrations of SNP. T compensates for the biological variations. The expts. were performed on symmetrical pieces, measurements of which are connected by lines. Note the logarithmic scale on the ordinate of



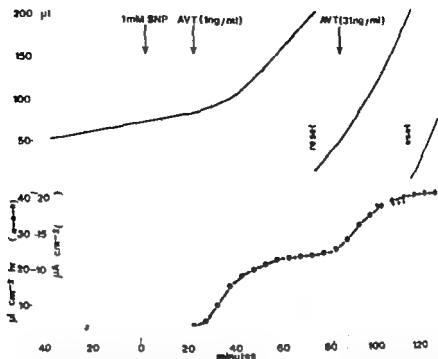


Fig. 1 Simultaneous measurements of waterflow and short circuit current using the apparatus described in the text for experimental details see Table 1. The upper curve shows the change with time of the volume of the closed half chamber (a 50  $\mu\text{l}$  syringe was used recorder speed was 2 mm/min). The bottom curve shows the first derivative of the upper curve taken each 5 min (0-0-0) calculated as  $\mu\text{l h}^{-1} \text{cm}^{-2}$ . Also shown is the short circuit current measured simultaneously (—). Reset the syringe was moved back to the start position and the volume was adjusted by means of a reservoir syringe.

Thus it seems possible that cGMP is able to modulate the response to hormonal stimulation of SCC and OWF. The authors mentioned above have used exogenous cGMP to increase the intracellular level a procedure which leaves one without any idea of what the intracellular level actually is. In the present paper we have used SNP which is known to stimulate cGMP accumulation in a number of tissues (Böhme et al 1978).

The purpose of this work has been to characterize the SNP induced cGMP accumulation in isolated frog skin epithelium and to investigate the relationship to the basal and the ADH stimulated SCC and OWF.

## METHODS AND MATERIALS

### Measurements of cGMP and cAMP content

The experiments were performed on female frogs (*Rana temporaria*). The frogs were kept partially immersed in tap water at 4°C. The skins were divided into 4 pieces, two and two symmetrical. The epithelia were isolated by incubation of the skin-pieces with collagenase in test tubes (polyethylene 100  $\times$  15 mm) containing 5 ml of modified

Ringer's solution (Na 115 mM, 5  $\text{Ca}^{2+}$ , 1  $\text{Mg}^{2+}$ , 1  $\text{Cl}^{-}$ , 118  $\text{CO}_2$ , 2.5  $\text{PO}_4$ , 1 glucose, 5 mM, pH=7.8). Exposure of both in- and outside of the skin to collagenase had no effect other than a minor decrease in responsiveness to stimulation with AVT reported earlier (Johnsen-Nielsen 1978). After 1.5 h of incubation with collagenase each skin-piece was placed on a glass plate and the intact epithelium was scraped off by means of a microscope slide. The epithelium was transferred to fresh medium in a new test tube. After 1 h equilibration the experiment was carried out under the desired conditions. During all incubations the test tubes were placed in a 37°C tube rotator.

After the incubation the epithelium was eluted between two brass blocks precooled in liquid  $\text{N}_2$ . The frog epithelium was homogenized with 500  $\mu\text{l}$  frozen 4% trichloroacetic acid (TCA) by a steel ball in a vibrating steel capsule (Mikro-dismembrator B, Braun Apparatebau). The TCA contained  $^3\text{H}$ -cGMP and  $^{14}\text{C}$ -cAMP as recovery markers. The steel capsule with content was precooled in liquid  $\text{N}_2$  and the temperature never exceeded 100°C during homogenization. After the homogenization, the capsule was recooled in liquid  $\text{N}_2$  and the powder transferred to a preweighed pyrex glass (75  $\times$  10 mm), where it was stored in liquid  $\text{N}_2$  until further processing. When desired the homogenate was allowed to thaw in an icebath and centrifuged for 10 min at 10,000  $g$  in the same tube. The precipitate was dried at 60°C and 100 mmHg.

#### Table 1. Responses to AVT after 20 min preincubation with 1 mM SNP

The equilibrium, symmetrical skin halves were preincubated for 20 min in the presence and absence of 1 mM SNP. AVT (1 ng/ml) was added to both skin halves. The stimulation of OWF and SCC was read 60 min later (values are mean  $\pm$  S.E. % increase  $100(a-b)/b$  where  $b$ =level before stimulation and  $a$ =level 60 min after stimulation) and the values between the two halves calculated. The maximal stimulating dose of AVT (31 ng/ml) was added to both halves and the stimulation of OWF and SCC read another 60 min later. The calculations were performed as mentioned above. Values are mean  $\pm$  S.E. With a  $t$ -test it was tested whether the ratios  $(+SNP-SNP)$  were different from 1.00.

ST	Pre-treatment	Unstimulated level	% increase after AVT			
			+SNP	-SNP	+SNP/-SNP	
1 ng/ml	OWF <sup>a</sup>	6.1 $\pm$ 0.5 $\mu$ l H <sub>2</sub> O/h cm <sup>2</sup>	254 $\pm$ 66	229 $\pm$ 71	1.31 $\pm$ 0.14 0.05 < P < 0.1	8
	SCC	13.9 $\pm$ 2.7 $\mu$ A/cm <sup>2</sup>	99 $\pm$ 34	97 $\pm$ 3	1.00 $\pm$ 0.04	
31 ng/ml	OWF	6.3 $\pm$ 0.6 $\mu$ l H <sub>2</sub> O/h cm <sup>2</sup>	483 $\pm$ 114	423 $\pm$ 9*	1.14 $\pm$ 0.03 0.005 < P < 0.01	6
	SCC	14.9 $\pm$ 3.6 $\mu$ A/cm <sup>2</sup>	134 $\pm$ 95	195 $\pm$ 82	0.67 $\pm$ 0.08 0.1 < P < 0.1	

<sup>a</sup> The percentage effect of SNP on the AVT induced OWF was smaller in the skins where AVT induced the larger response in OWF. This explains the discrepancy between the ratio of the means (254/229=1.11) and the mean of the ratios for the individual experiments (1.31).

period. After a lag period about 1 min the cGMP increased approximately 10 times within the next 4 min and more slowly the following 20 min. 0.1 mM SNP induced a small (23% in average,  $n=3$ ) increase in cAMP level within the first minute (Fig. 3a), after which the level was constant for the next 30 min. From Fig. 2a, periods of 5 min duration were chosen as suitable for examining the dose-response relationship. 0.01 mM SNP caused a significant increase in the cGMP level and a maximum was reached with 1 mM (Fig. 3). This series confirms that SNP had only a small effect on cAMP increase for 5 min with 0.01 mM SNP caused a

20% increase in cAMP level (Fig. 3b) while the step from 1 to 10 mM resulted in a 13% decrease ( $n=4$ ).

#### Effect of SNP on OWF and SCC

In these expts. 1 mM SNP was used since this concentration gave maximal increase of the cGMP level and had negligible effect on the cAMP level. As seen from Table 1 1 mM SNP had no effect on OWF or SCC during a 20 min period. Neither did expts. with longer incubation periods or lower concentrations of SNP reveal any effects on OWF or SCC (data not shown).

#### Table 2. cAMP response to AVT after 20 min preincubation in 1 mM SNP

Symmetrical pieces were incubated for 20 min in solution with and without 1 mM SNP then AVT (1 or 40 ng/ml) was added to both pieces and cAMP measured in both 5 and 30 min later. Values are mean  $\pm$  S.E.

ST	Time	pmole cAMP/mg dry weight			
		SNP	-SNP	+SNP/-SNP	
1	5	1.16 $\pm$ 0.09	1.28 $\pm$ 0.12	0.93 $\pm$ 0.07	4
	30	1.37 $\pm$ 0.26	1.57 $\pm$ 0.36	0.90 $\pm$ 0.05	4
40	5	6.47 $\pm$ 1.11	7.68 $\pm$ 2.24	n.s.	5
	30	60 $\pm$ 0.83	2.13 $\pm$ 0.65	0.90 $\pm$ 0.08 n.s. 1.20 $\pm$ 0.05 0.01 < P < 0.02	5

1. not significant.

Table 1 *Effect of SNP on OWF and SCC*

Paired halves were allowed to equilibrate and then 1 mM SNP was added to one half (time 0). OWF and SCC were re-time 0 and 20 min and the change with time calculated as the difference between the values at the two times ( $\Delta$ ). Difference between the changes of the control-half ( $\Delta -$ ) and the experimental half ( $\Delta +$ ) were calculated and taken as effect of SNP. Values are mean  $\pm$  S.E.

Parameter	SNP	Minute		$\Delta$	$\Delta + - \Delta -$	n
		0	20			
OWF	+	$6.6 \pm 0.8$	$7.1 \pm 1.0$	$0.5 \pm 0.3$	$0.1 \pm 0.3$	11
$\left(\frac{\mu\text{I H}_2\text{O}}{\text{h cm}}\right)$	-	$6.3 \pm 0.6$	$6.7 \pm 0.8$	$0.4 \pm 0.4$		
SCC	+	$13.0 \pm 2.7$	$11.8 \pm 2.3$	$-1.1 \pm 0.5$	$0.1 \pm 0.3$	11
$(\mu\text{A}/\text{cm}^2)$	-	$13.3 \pm 2.5$	$12.0 \pm 2.4$	$-1.2 \pm 0.5$		

the specificity of the cGMP antiserum was satisfactory. The authenticity of the measured cGMP values was verified by carrying standards through the extraction and purification procedure and by internal standards and dilution curves of epithelial extracts.

In one series (Table 3) only cAMP was measured and the more convenient boiling method (Johnsen & Nielsen 1978) was used with the modification that the homogenization was replaced by a mixing on a whirl-mixer. In a control series it was found that mixing gave  $4 \pm 1\%$  ( $n=6$ ) higher values than did homogenization.

#### Measurements of SCC and ONF

The hydroosmotic response to AVT of *R. temporaria* was found to be rather small compared to that of *R. esculenta*. 31 ng/ml AVT increased the OWF 50–100% in *R. temporaria* vs 700–800% in *R. esculenta*. Therefore *R. esculenta* was used for the measurement of OWF and SCC. The *R. esculenta* were kept at room temperature with free access to water and food. A few experiments confirmed that the SNP induced cGMP accumulation in *R. esculenta* is similar in magnitude to the one in *R. temporaria*.

The OWF measurements were carried out in a chamber which allows the simultaneous measurements of SCC and OWF (SCC was measured according to the technique of Ussing & Zerahn (1951)). This chamber is improved from the one described by Lichtenstein & Leaf (1965) with an automatic recording device akin to that described by Ruppel et al. (1972). In short the principle is that the outside (apical side) of the skin is pressed against a stainless steel net with a pressure of 2 cm H<sub>2</sub>O. The half chamber containing the outside bathing solution is closed except for an outlet consisting of a capillary tube. The outside bathing solution is allowed to flow into the capillary tube whereby the light transmission of the tube changes markedly. The transmission is recorded by a detection fork (Hafslund) (consisting of a light emitting diode and a photosensitive transistor) the signal of which controls a motor driven syringe to keep the position of the meniscus constant. The motor also drives a precision potentiometer which is used as a potential divider allowing the position of the syringe

to be recorded continuously on a pen recorder. The slope of the recording then gives the flow rate (Fig. 1).

The detection limit of the apparatus was determined by changing the volume of a closed half chamber by means of a 1  $\mu$ l syringe and it was found to be better than 50 nl overall precision of the flow rate determination depends upon detection limit, recorder speed and size of the driven syringe. In the present experiments a 50  $\mu$ l syringe (Hamilton) was used and the recorder speed was 2 cm/min this gave a precision better than 0  $\mu$ l h<sup>-1</sup> cm when the flow was 5  $\mu$ l h<sup>-1</sup> cm (typical basal flow) a precision better than 0.5  $\mu$ l h<sup>-1</sup> cm when the flow was 25  $\mu$ l h<sup>-1</sup> cm (typical stimulated flow).

## Material

Synthetic AVT the neurohypophyseal hormone from *Xenopus laevis* was obtained from Calbiochem.  $\text{AlO}_3$  90 active was obtained from Merck. SNP (Merck) was dissolved immediately before use.  $^{32}\text{P}$ -cGMP (1 Ci/mmol),  $^3\text{H}$ -cAMP (10 Ci/mmol) and  $^{32}\text{P}$ -cGMP (6 Ci/mmol) was obtained from Amersham. cGMP standards were kindly supplied by J. Arends, Statens Seruminstitut, Copenhagen.

## RESULTS

Paired symmetrical pieces were used for all experiments throughout this paper in order to compensate for the rather large biological variations in frog skin.

### Effect of SNP on cGMP and cAMP

Basal level of cGMP was  $14.7 \pm 1.7$  [fmol cGMP/mg dry weight (mean  $\pm$  S.E.  $n=16$ )]

Incubation with 0.1 mM SNP caused a marked increase in the cGMP level ( $F = 2.2$ , the test for

*Table 2. Responses to AVT after 30 min preincubation in 1 mM SNP*

On the application, symmetrical skin halves were preincubated for 30 min in the presence and absence of 1 mM SNP. Under AVT (1 ng/ml) as added to both skin halves. The stimulation of OWF and SCC was read 60 min later (values are  $\bar{x} \pm s$ ,  $n=10$  ( $a-b$ ), here  $b$ =level before stimulation and level 60 min after stimulation), and the increase in the two halves calculated. Then a maximal stimulating dose of AVT (31 ng/ml) was added to both halves and the stimulation of OWF and SCC read another 60 min later. The calculations were performed as mentioned above. Values are mean  $\pm$  S.E. Each  $t$ -test it was tested whether the ratios  $(\bar{S}NP - \bar{S}NP)$  were different from 1.00.

AVT	Parameter	Unstimulated level	% increase after AVT			
			+SNP	-SNP	+SNP/-SNP	
1 ng/ml	OWF	$6.1 \pm 0.5 \mu\text{l H}_2\text{O}/10 \text{ cm}^2$	$254 \pm 66$	$229 \pm 71$	$1.31 \pm 0.14$ $0.05 < P < 0.1$	3
	SCC	$13.9 \pm 2.7 \mu\text{A}/\text{cm}^2$	$99 \pm 34$	$97 \pm 32$	$1.00 \pm 0.04$	
10 ng/ml	OWF	$6.3 \pm 0.6 \mu\text{l H}_2\text{O}/10 \text{ cm}^2$	$485 \pm 114$	$423 \pm 97$	$1.14 \pm 0.03$ $0.005 < P < 0.01$	6
	SCC	$14.9 \pm 3.6 \mu\text{A}/\text{cm}^2$	$154 \pm 55$	$195 \pm 87$	$0.87 \pm 0.08$ $0.1 < P < 0.2$	

The percentage effect of SNP on the AVT induced OWF was smaller in the skins where AVT induced the larger response OWF. This explains the discrepancy between the ratio of the means ( $254/229 = 1.11$ ) and the mean of the ratios for the individual experiments (1.31).

After a lag period about 1 min the cGMP level was approximately 10 times within the next 4 min, and more slowly the following 20 min. 0.1 mM SNP induced a small (23% in average  $n=3$ ) increase in cAMP level within the first minute (Fig. 3a), after which the level was constant for the next 30 min. From Fig. 2 periods of 5 min duration were chosen as suitable for examining the dose-response relationship. 0.01 mM SNP caused a significant increase in the cGMP level and a maximum was reached with 1 mM (Fig. 3a). This series confirms that SNP has only a small effect on cAMP stimulation for 5 min with 0.01 mM SNP caused a

20% increase in cAMP level (Fig. 3b) while the step from 1 to 10 mM resulted in a 13% decrease ( $n=4$ ).

*Effect of SNP on OWF and SCC*

In these expts. 1 mM SNP was used since this concentration gave maximal increase of the cGMP level and had negligible effect on the cAMP level. As seen from Table 1 1 mM SNP had no effect on OWF or SCC during a 20 min period. Neither did expts. with longer incubation periods or lower concentrations of SNP reveal any effects on OWF or SCC (data not shown).

*Table 3. cAMP response to AVT after 20 min preincubation in 1 mM SNP*

Symmetrical pieces were incubated for 20 min in solution with and without 1 mM SNP then AVT (1 or 40 ng/ml) was added to both pieces and cAMP measured in both 5 and 30 min later. Values are mean  $\pm$  S.E.

AVT	Time	pmoles cAMP/mg dry weight			
		SNP	-SNP	+SNP/-SNP	
1 ng/ml	5	$1.16 \pm 0.09$	$1.28 \pm 0.12$	$0.93 \pm 0.07$	4
	30	$1.57 \pm 0.76$	$1.57 \pm 0.36$	$0.90 \pm 0.05$	4
40 ng/ml	5	$6.47 \pm 1.11$	$7.68 \pm 2.24$	$0.90 \pm 0.08$	5
	30	$2.40 \pm 0.83$	$2.13 \pm 0.65$	$1.20 \pm 0.03$ $0.01 < P < 0.02$	5

$n.s.$  not significant

### Effect of SNP on stimulation with AVT

To test whether SNP influenced the ability of ADH to stimulate OWF and SCC a series of expts were performed where the skins were preincubated for 20 min in the presence and absence of SNP. After the preincubation a submaximal dose of AVT (1 ng/ml) was added and the SCC and OWF were recorded for 60 min. Then a dose giving maximal stimulation of SCC (31 ng/ml) (Johnsen & Nielsen 1978) was added and the SCC and the OWF were recorded for another 60 min. SNP had no significant influence on the AVT induced stimulation of SCC but it enhanced the stimulation of OWF moderately (Table 2). This potentiation was significant at the high concentration of AVT. At the low concentration of AVT SNP was found to potentiate the AVT induced increase of OWF in 7 out of 8 expts. In these 7 expts the mean value of the ratio between OWF in the presence and absence of SNP was  $1.38 \pm 0.14$  ( $0.025 < P < 0.05$ ).

To make sure that 31 ng/ml AVT induces maximal stimulation of OWF and SCC the following series of 3 expts was performed. At first the skins were stimulated by 31 ng/ml AVT and when a new steady state was reached the concentration of AVT was increased to 186 ng/ml. This increase in the AVT concentration stimulated the SCC by 19.07 and 2.4% and the OWF by 28.0 and 0%.

To investigate whether the effect of SNP on the AVT induced stimulation of OWF could be attributed to a change in the cAMP level the expts in Table 3 were performed. Stimulation with AVT results in an increase of the cAMP level which reaches a maximum after 5 min thereafter it declines to a semi-steady state level at 30 min (Johnsen & Nielsen 1978). Since it is not known whether the magnitude of the physiological responses SCC and OWF depends on the peak or the steady state value of the cAMP level measurements were made 5 and 30 min after addition of AVT. Preincubation with SNP had only a significant effect on the AVT stimulated cAMP accumulation in the series carried out at the high AVT concentration for 30 min where a slight potentiation by SNP was found (Table 3).

### DISCUSSION

SNP stimulates cGMP accumulation in a number of tissues (Böhme et al 1978). In the present com-

munication it is shown that SNP causes a marked increase in the cGMP level in the whole epithelium of frog skin (Fig. 2a and Fig. 2b) whereas SNP has only a negligible effect on cAMP level (Fig. 2b and Fig. 3b). The SNP induced cGMP accumulation in frog skin is comparable in magnitude and concentration dependence to the one found in smooth muscle tissue (Böhme et al 1978).

SNP per se has no effect on OWF or SCC (Table 1) indicating that intracellular cGMP has no effect on either of these parameters. But SNP has a moderate potentiating effect on the AVT induced stimulation of OWF while it is without effect on stimulation of SCC (Table 2). SNP also enhances the elevation of the semi-steady state level of cAMP induced by a dose of AVT (31 ng/ml) that gives maximal stimulation of SCC and OWF (Table 3). It is tempting to use this as an explanation of the potentiating effect of SNP on the OWF stimulation by AVT. But this is probably not the case since hormone doses exceeding those needed for maximal physiological stimulation are generally capable of eliciting a further increase in the cAMP level (Robinson et al 1971). In toad bladder for example ADH concentrations higher than necessary to give maximal stimulation of SCC and OWF produce additional cAMP accumulation (Flores et al 1977). Thus it seems more likely that the potentiation of the response to AVT by SNP comes from interference of cGMP or SNP with another step in the stimulus response coupling than the stimulation of adenylyl cyclase.

Our results are in contrast to the findings of Piccini & Paris (1973) that exogenous cGMP inhibits the stimulation of OWF by oxytocin in toad bladder. Unless this discrepancy is due to species differences the result of Piccini & Paris might be a nonspecific result of the high concentration of cGMP (10 mM) employed in their expts. Sugita et al (1973) found a small potentiating effect of exogenous cGMP on the SCC response to ADH in toad bladder whereas we found a potentiating effect of SNP on the OWF response in frog skin.

The conclusion to be drawn from the expts presented in this paper is that cGMP in contrast to cAMP has no influence on OWF or SCC and plays no part in the regulation of SCC by AVT. On the other hand sodium nitroprusside moderately potentiates the AVT stimulation of OWF. This may be due to the enhanced cGMP level but it cannot be

added that it is caused by an effect of SNP on the parameters. Our results lends no support to the general idea of opposing effects of cAMP and cGMP (Goldberg et al. 1972). The idea of opposing effects of cAMP and cGMP was recently supported by the observation that carbamylcholine reduces the SCC and increases the cGMP level in toad bladder (Wessman et al. 1978; Sahib et al. 1978). Considering our results it seems more likely that the carbamylcholine induced increase in cGMP level does not induce the reduction of SCC. This possibility is also mentioned by Wessman et al. (1978) as they have suggested that both the decrease in SCC and increase in cGMP level induced by carbamylcholine are elicited by the entry of calcium into the cells.

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# Functional properties of neurones in the parietal retroinsular cortex in awake monkey

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The parietal retroinsular cortex of three awake behaving macaque monkeys was investigated using transdural microelectrode recording technique. All 73 cells identified responded to somatosensory stimuli. Most of the neurones (51) were activated by compression of the skin in an on, off or on-off fashion; these cells were unresponsive to light touching or stroking of the skin. The rest of the cells responded to light touching, rotation of a joint or palpation of a muscle belly. All body parts were represented in this area. Of the cells 30% responded to stimulation of both sides of the body. The results indicate that the parietal retroinsular cortex participates in the analysis of skin compression. This information is used, e.g. in the control of manipulative movements and in discrimination of supported rights. It is possible that ablation of the area examined would impair these functions.

**Key words:** Parietal lobe, somatosensory cortex, microelectrode recording.

In SII of the monkey buried in the medial wall of the Sylvian fissure was previously investigated by single cell recording technique by Whitsel et al (1969). Their study did not include the most posterior part of the medial wall. All of the Sylvian fissure cells they regarded as part of SII. However, there is histological evidence (Pandya & Smailes 1973; Jones & Barak 1976) that the parietal retroinsular cortex in the caudal end of the fissure is architecturally different from SII. The aim of this study was to explore this part of the parietal retroinsular cortex and to compare its functional properties with those documented from SII by Whitsel et al (1969). All cells in this study were responsive to somatosensory stimulation, but in contrast to cells in SII most of them did not respond to light touching or stroking of the skin but only to compression (causing clear deformation) of the skin.

preparation. The discharges of each neurone isolated were observed during the monkey tracing and fixating eye movements when the monkey reached and grasped objects under visual or tactual guidance or when the monkey brought objects to the mouth. Each neurone was also tried to activate by light touch, pressure on the skin (compression of the skin between the underlying bone and the external pressure), palpation of muscle bellies, rotation of joints and various moving and stationary visual stimuli. Fig. 1 shows the area recorded and the histological map by Pandya & Smailes (1973) of the posterior part of the Sylvian fissure and temporal lobe.

## RESULTS

All cells identified in the retroinsular cortex responded only to somatosensory stimulation. Properties of only one of the 74 cells studied remained unidentified (Table 1).

*Cells responding to touch or pressure on the skin*

8 of the 59 cells responsive to cutaneous stimulation were activated by light touch on the skin or blowing into the hair. Of the cells 51 responded only to compression of the skin (causing clear deformation) usually in an on-off or on-off fashion.

**MATERIAL AND METHODS**  
Recordings were made from four hemispheres of two adult male and one juvenile male Macaca species monkeys weighing 6-8 kg. Records obtained from the same monkeys were from 7 and the temporoparietal association cortices are reported elsewhere (Leinonen 1980; Leinonen et al. in



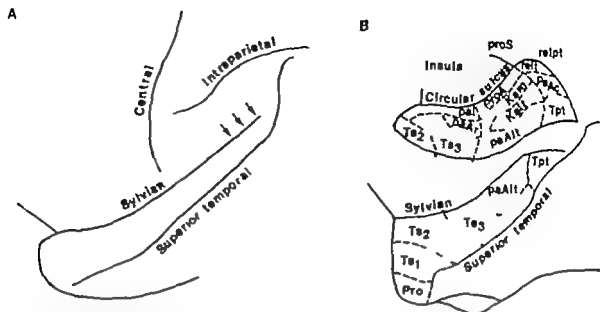


Fig 1 A The sulci of the parietal lobe and the recording site of the present work in the Sylvian fissure (arrows). Cytoarchitectonic map by Pandya & Sanides (1973) of the posterior part of the Sylvian fissure and the superior temporal gyrus. Area relpt is the parietal retroinsular area recorded.

(Fig. 2) Some cells fired for several seconds during continual pressure on the skin (Fig. 3) 37 cells had a receptive field on the hand or arm 5 cells on the shoulder neck or head 2 on the trunk and 15 on the legs only (Fig. 4). The receptive fields were rather large e.g. the whole hand whole foot all fingers whole arm. Those cells that had a receptive field on the hand were usually active also when the monkey was manipulating an object or grooming.

#### Cells responding to rotation of joints or palpation of muscles

Fourteen cells responded to forced or rapid dorsal flexion of fingers or wrist (5 cells) dorsal flexion of the ankle joint (7 cells) or wrist and ankle joints (1 cell) to palpation of muscle bellies in the lower or upper arm (7 cells) or legs (1 cell) to passive rotation of the back or neck and to palpation of the muscles of the neck or back (3 cells) (Fig. 4).

#### Lateralization

Of the 73 cells 51 had their receptive field on contralateral side of the body 22 cells were activated by stimuli on either side of the body. All the bilateral receptive fields were on the extremities the responses were usually equally strong to stimuli on the right and left sides.

#### Referent body part

All parts of the body were represented in the area examined. Most cells (48 out of 73) had a receptive field on the hand or arm the rest on the shoulder neck head trunk or legs.

#### DISCUSSION

Area SII of the monkey buried in the Sylvian fissure was studied with single cell recording technique.

Table 1 Classification of the cells in the parietal retroinsular cortex according to the type of effective stimulus

	No. of cells
Cell responding to touch or pressure on the skin	59
Cell responding to palpation of muscles or rotation of joint	11
Not identified	1
Total	74



Fig 2 On-responses of a cell to compression of the contralateral (A) and ipsilateral arm (B). Response to contralateral stimuli lasted longer than responses to ipsilateral stimuli. The cell had no spontaneous activity.



Fig. 3. Response of cell to compression of the contralateral forefinger. The response lasted several seconds during stationary stimulus. No off-responses were observed. The cell had no spontaneous activity.

by Whitsel et al. (1969). This study did not include the most posterior part of the Sylvian fissure. However, Whitsel et al. proposed that SII extends to the end of the fissure, which view is also held by Woolsey (1948) and Roberts & Allett (1963). In their histological investigations Pandya & Pandya (1973) and Jones & Burton (1976) described the retroinsular area in the dorsal wall of the Sylvian fissure posterior to SII and lateral to area 7. The

present study was conducted in this area. The comparison of the results from the retroinsular area with those obtained from SII by Whitsel et al. (1969) shows that the areas are functionally different. The areas are similar as far as all cells respond to somatosensory stimulation and all parts of the body are represented in both areas. The findings differ in the following respects. (1) In SII 87% of the cells responded to light touching or stroking of the skin and 13% were activated only by compression causing clear deformation of the skin whereas in the retroinsular area only 10% of the cells responded to light touch or stroking and most cells were activated only by compression of the skin or palpation of muscle bellies or rotation of joints. (2) In SII 90% of the cells had bilateral receptive fields whereas in the retroinsular cortex most (70%) cells had only contralateral receptive fields.

#### *Connections of the retroinsular cortex*

Burton & Jones (1976) showed that the anterior and medial parts of the posterior nucleus of the thala-

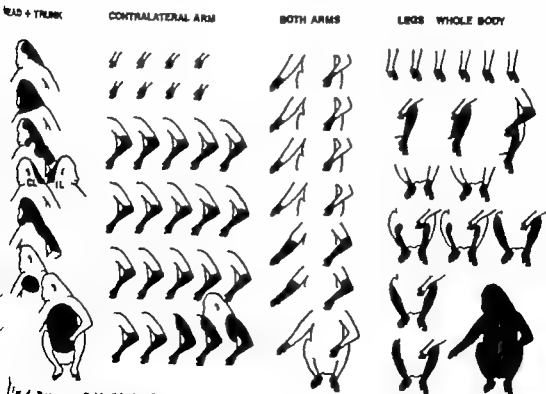


Fig. 4. Receptive fields (black) of all cells responsive to cutaneous stimulation. The receptive fields on the extremities extend around the limb.

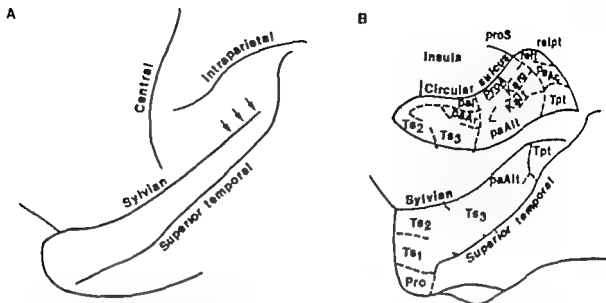


Fig. 1. A: The sulci of the parietal lobe and the recording site of the present work in the Sylvian fissure (arrows). B: Cytoarchitectonic map by Pandya & Sanides (1973) of the posterior part of the Sylvian fissure and the superior temporal gyrus. Area relpt is the parietal retrolateral area recorded.

(Fig. 2). Some cells fired for several seconds during continual pressure on the skin (Fig. 3). 37 cells had a receptive field on the hand or arm, 5 cells on the shoulder, neck or head, 2 on the trunk and 15 on the legs only (Fig. 4). The receptive fields were rather large, e.g. the whole hand, whole foot, all fingers, whole arm. Those cells that had a receptive field on the hand were usually active also when the monkey was manipulating an object or grooming.

#### Cells responding to rotation of joints or palpation of muscles

Fourteen cells responded to forced or rapid dorsal flexion of fingers or wrist (5 cells), dorsal flexion of the ankle joint (2 cells) or wrist and ankle joints (1 cell), to palpation of muscle bellies in the lower or upper arm (2 cells) or legs (1 cell), to passive rotation of the back or neck and to palpation of the muscles of the neck or back (3 cells) (Fig. 4).

#### Lateralities

Of the 73 cells, 51 had their receptive field on contralateral side of the body. 2 cells were activated by stimuli on either side of the body. In the bilateral receptive fields were on the extremities, the responses were usually equally strong to stimuli on the right and left sides.

#### Referent body part

All parts of the body were represented in the area examined. Most cells (48 out of 73) had a receptive field on the hand or arm, the rest on the shoulder, neck, head, trunk or legs.

#### DISCUSSION

Area SII of the monkey, buried in the Sylvian fissure, was studied with single cell recording technique.

Table 1. Classification of the cells in the parietal retrolateral cortex according to the type of effective stimuli

	No. of cells	n
Cells responding to touch or pressure on the skin	59	80
Cells responding to palpation of muscles or rotation of joint	14	19
Not identified	1	1
Total	74	100



Fig. 2. On-responses of a cell to compression of the contralateral (A) and ipsilateral arm (B). Responses to contralateral stimuli lasted longer than response to ipsilateral stimuli. The cell had no spontaneous activity.

# Diurnal fluctuation in striatal choline acetyltransferase activity and strain difference in brain protein content of the rat

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NORDBERG A & WAHLSTRÖM G: Diurnal fluctuation in striatal choline acetyltransferase activity and strain difference in brain protein content of the rat. *Acta Physiol Scand* 1980, 108, 385-388. Received 13 July 1979. ISSN 0001-6772. Department of Pharmacology, University of Uppsala, Sweden and Department of Pharmacology, University of Umeå, Sweden.

Choline acetyltransferase (CAT) activity was measured in two brain regions of rat at different times of the day. A diurnal fluctuation of the CAT activity was found in the striatum with high activity during the light period and low activity during the dark period. In the hippocampus + cortex the CAT activity remained constant. A marked difference in the brain protein content was found between two strains of Sprague-Dawley rats. This finding emphasizes the importance of expressing enzyme activity both per g tissue and g protein especially when comparing data between different research groups.

**Key words.** Choline acetyltransferase, diurnal fluctuation, rat, brain regions, strain.

A diurnal fluctuation in the content of acetylcholine (ACh) in brain has been reported by several research groups (Friedman & Walker 1969; Hansen *et al.* 1970; Saito 1971; Saito *et al.* 1975; Massarelli 1975). There are also data indicating that the enzyme choline acetyltransferase (CAT) responsible for the synthesis of ACh may fluctuate in activity with diurnal periodicity in the brain (Walker & Spence 1975; Massarelli 1975). In the present study we present data indicating that the activity of CAT in the striatum of rat fluctuates diurnally while remaining constant in the hippocampus and cortex. In addition a marked difference in brain protein content was measured between two strains of rats.

## METHODS

Two strains of male Sprague-Dawley rats were used namely Møl/Møl/Møl Möllegaard L, Skerfvej, Denmark (04-415 g) (strain A) and Møl/Møl/Anticimex, Stockholm, Sweden (370-475 g) (strain B). The animals were kept 3/age. The artificial light which was the only light source was for strain A on an inverted schedule with light between 8.00 p.m. and 8.00 a.m. For strain B the light was between 6.00 a.m. and 6.00 p.m. Food and water were

available *ad libitum*. For the study of the diurnal fluctuation in CAT activity only rats of strain B were used and they were kept under our diurnal lighting pattern for at least 2 months before they were killed.

At the time of sacrifice each rat was separately removed from the animal room and immediately killed in room dark by the animals were killed by decapitation. The brains were rapidly removed and put on an ice-cold glass plate. The striatum and the hippocampus + cortex were dissected essentially according to the method described by Glowinski & Iversen (1965). The tissues were homogenized in solution of NaCl 150 mM-EDTA 0.2 mM-butanol 1% (1:100 w/v). The activity of CAT was measured according to the method by Foonara (1969) with some modifications (Glover & Green 1972; Aquasolines *et al.* 1975). 10 µl of the homogenate were incubated with 10 µl phosphate buffer 20 mM (pH 7.0) containing <sup>3</sup>H-acetyl-CoA (100 mCi/mmol) (40 µM), choline (Ch) Cl 10 mM, KCl 200 mM and physostigmine subtylate 0.2 mM for 10 min at 37°C. The reaction was stopped by adding 10 µl 1 M HAc. Radioactive ACh (<sup>3</sup>H ACh) formed was extracted into 100 µl 3-heptanone containing 0.1 M K<sub>2</sub>Hg<sub>4</sub>. Fifty microliters of the organic layer were added to a scintillation cocktail containing toluene, naphthalene, Permafluor 111 and the radioactivity was measured. The protein content of the homogenates were measured according to Lowry *et al.* (1951). Bovine albumin was used as standard.

mus project to the parietal retroinsular area in the posterior part of the Sylvian fissure. They also stated that "Projections from the ventroposterior nucleus outline the boundaries of the second somatic sensory area in the upper bank of the lateral sulcus. The posterior boundary of II appears to contain the representation of the hind limb and abuts mainly upon the retroinsular field and area 7 but does not overlap into either of these". The posterior nucleus of the thalamus is thus one possible source of somesthetic information received by the retroinsular area. Information may also come via SI and area 5 which project to the area studied (Jones & Powell 1970). The whole dorsal wall of the Sylvian fissure receives transcallosal fibers (Pandya et al 1971) which is in accordance with the finding that 30% of the cells examined had bilateral receptive fields.

#### Role of the parietal retroinsular cortex

Burton & Jones (1976) have discussed the projection from the posterior thalamus to the parietal retroinsular cortex in the light of animal experiments and clinical findings on central mechanisms of pain. The present study revealed that most cells in the parietal retroinsular cortex responded only to rather intense pressing of the skin. However, if e.g. the monkey's withdrawal of the hand were considered as a criterion for a painful stimulus the stimuli used were not painful.

Pandya & Sanides (1973) suggested that the parietal retroinsular field receives vestibular projections. Burton & Jones (1976) proposed that vestibular projections from the posterior thalamus terminate in the dysgranular insular area. The present study showed that all cells in the retroinsular cortex responded to somatosensory stimuli. It was not examined whether they would have responded also to vestibular stimuli. However, the results show that this area is not purely a vestibular projection field which agrees with the concept of Burton & Jones (1976) about the vestibular projection area.

In the parietal retroinsular area most cells examined responded to compression of the skin. Similar stimuli have been observed to activate cells of area 7 near the retroinsular area (Leinonen 1980). Cells in the bordering part of area 7 respond also to visual stimuli or are active during the monkey's own movements, most often during movements of the hands, e.g. grasping, manipulation and grooming.

It is possible that information from the parietal retroinsular area is transferred to area 7 where it is used for the control of hand movements. The information analyzed in the parietal retroinsular might also be used for discrimination of support weights, which ability has been shown to deteriorate after a damage in the posterior parietal cortex (Ruch et al 1978).

The technical assistance of Riitta Kerttunen, Tuula Mäkinen and Ilkka Linnankoski is gratefully acknowledged. I also thank Prof. Johani Hyvärinen for the support given during the work and for critical comments on manuscript. This work was supported by grants from Academy of Finland Research Council for Medical Sciences and from Emil Aaltonen foundation.

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Table 1. CAT activities in the striatum and hippocampus + cortex of 2 different strains of Sprague-Dawley rats

Mean value  $\pm$  the standard error are given. n denotes number of rats. In all statistical comparisons strain A is compared with strain B.

	nmol ACh/g tissue/min	nmol ACh/mg protein/min	mg protein/g tissue
<b>Striatum</b>			
Strain A	122.13 $\pm$ 4.75** n=9	3.19 $\pm$ 0.4 n=8	39.18 $\pm$ 2.10* n=8
Strain B	234.88 $\pm$ 15.5* n=9	3.68 $\pm$ 0.29 n=5	63.9 $\pm$ 3.02 n=7
<b>Cortex + hippocampus</b>			
Strain A	86.64 $\pm$ 1.67** n=9	1.48 $\pm$ 0.07 n=7	57.96 $\pm$ 1.95*** n=7
Strain B	119.16 $\pm$ 8.35 n=9	1.38 $\pm$ 0.11 n=5	74.86 $\pm$ 2.88 n=5

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

killed by decapitation while no difference in ACh content was found following freezing in liquid nitrogen. Saito et al. (1975) also recorded higher ACh values in rats killed during the light period in comparison with the dark period but in contrast to Hansen et al. (1970) this diurnal rhythm in ACh content was found independent of the method of sacrifice. Recently it has been shown that killing animals with microwave irradiation is necessary in order to prevent post mortem changes in ACh levels (Stavinos et al. 1973; Nordberg & Sundwall 1976). As far as we know, no study concerning diurnal ACh fluctuation following this rapid method of killing has been presented.

In agreement with the findings concerning ACh we found in the present study a high CAT activity during the light period and a lower activity during the dark period. It is surprising to find this since CAT is thought to be non-rate limiting in the synthesis of ACh (Kuhar & Murrin 1978). But the fact that this diurnal fluctuation in CAT only could be found in the striatum and not in the cortex + hippocampus might be explained by the very rapid turnover and high concentration of ACh in the striatum in comparison with other brain regions (Nordberg 1977). Since the previous reports on changes in CAT activity mentioned in the introduction (Walter & Ikpoodia 1975; Massarelli 1975) are not given with sufficient details a comparison with the present material is not possible. In the abstract by Walter & Ikpoodia (1975) it is not stated at what time the light was switched off. Massarelli (1975)

used the whole brain of the mouse. Recently data have been presented indicating a higher CAT activity in the cerebral cortex of humans who have died in the morning compared with during the night (Perry et al. 1977a, 1977b). Whether this finding represents a diurnal fluctuation in CAT activity is hard to decide on this material only since the status of the individuals before and at the time of death might influence the results and the number of patients in each time group is small.

This study was supported by the Swedish Medical Research Council (project 2879/3771) and the Swedish Tobacco Company. The technical assistance of Miss Maria Astm, Mr Roland Larsson, Mrs Elisabeth Ljungblad and Mrs Kerstin Wahlström is thankfully acknowledged.

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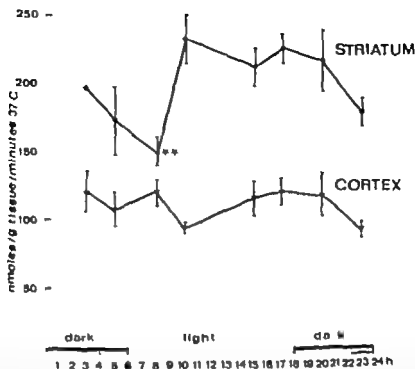


Fig. 1. Diurnal fluctuation in CAT activity (nmol formed ACh/g tissue/min) in the striatum and cortex (including hippocampus). Each point represents the mean value of 4 rats. Vertical bars indicate S.E.  $P < 0.05$ ,  $P < 0.01$  in comparison with CAT activity at 10 a.m.

## RESULTS

Table 1 shows the activity of CAT in the striatum and cortex + hippocampus of two different strains of Sprague-Dawley rats. The rats were killed between 1 h before and 7 h after the light had been switched off. A significantly lower CAT activity expressed in nmol ACh formed/g brain tissue/min was found in the striatum and cortex + hippocampus of strain A in comparison with strain B. In contrast, no difference in enzyme activity was found between strain A and B when the activity was expressed in nmol/mg protein/min. As shown in table 1 this finding can be explained by significantly lower protein content/g tissue in the striatum ( $-63\%$ ) and cortex + hippocampus ( $-79\%$ ) of strain A in comparison with strain B.

Fig. 1 shows the CAT activity (nmol/g/min) in the striatum and cortex + hippocampus of rats killed at different time of the day. No significant difference in the CAT activity was found in the cortex + hippocampus. However, in the striatum a diurnal fluctuation in CAT activity was recorded. A sharp rise in the enzyme activity was measured 4 h after the light had been switched on (at 10 a.m.). The CAT activity then was rather constant during the

rest of the light period and dark period until 11 p.m. (5 h dark) when the CAT activity was significantly lower ( $-74\%$ ) in comparison with the activity at 1 p.m. The activity continued to be low during the rest of the dark period and at 7 h after the light had been switched on again (8 a.m.) the lowest CAT activity was measured ( $-35\%$  in comparison with the value at 10 a.m.). Between 8 a.m. and 10 a.m. there was a marked increase in the CAT activity. Thus the curve is essentially biphasic with a distinct maximum and minimum. The same diurnal fluctuation in CAT was obtained when the enzyme activity was given in nmol ACh synthesized/mg protein/min. No diurnal fluctuation in protein content of the brain was found.

## DISCUSSION

Hanin et al. (1970) found a diurnal oscillation in the content of ACh of rat brain. The ACh content was highest after 7 h light and lowest after 6 h darkness. The fluctuation in the ACh content was only observed in grouped animals which had been under controlled environment with 12 h light and 12 h dark for at least 18 days. It could only be observed in rat

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<sup>a</sup>  $P < 0.01$  <sup>b</sup>  $P < 0.001$

killed by decapitation while no difference in ACh content was found following freezing in liquid nitrogen. Saito et al. (1975) also recorded higher ACh values in rats killed during the light period in comparison with the dark period but in contrast to Hatanaka et al. (1970) this diurnal rhythm in ACh content was found independent of the method of sacrifice. Recently it has been shown that killing animals with microwave irradiation is necessary in order to prevent post-mortem changes in ACh levels (Sundin & Nordberg 1973; Nordberg & Sundin 1976). As far as we know no study concerning diurnal ACh fluctuations following this rapid method of killing has been presented.

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# Capillary permeability in canine myocardium as determined by bolus injection detection

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HAUNSO S., PAASKE W. P., SEJRSEN P. & AMTORP O. Capillary permeability in canine myocardium as determined by bolus injection residue detection. *Acta Physiol Scand* 1980, 108: 389-397. Received 15 July 1979. ISSN 0001-6772. Department of Cardiology, Copenhagen County Hospital Gentofte, Department of Internal Medicine P, Ryghøjsgade, and Institute of Medical Physiology B, University of Copenhagen, Denmark.

Capillary permeability of  $^{51}\text{Cr}$  EDTA in the canine myocardium was determined by applying: (A) the single injection, external registration method, and (B) the local tissue clearance method to the intact dog heart of open-chest anesthetized dogs. (A)  $^{51}\text{Cr}$  EDTA was administered into the left anterior descending coronary artery as a bolus injection, and the response curve was recorded by external registration. The capillary diffusion capacity (the permeability-surface area product) for  $^{51}\text{Cr}$  EDTA amounted to  $32.5 \text{ ml}/100 \text{ g min}$  at capillary extraction of 0.40 and a plasma flow of  $73.3 \text{ ml}/100 \text{ g min}$ . The diffusional permeability coefficient of  $^{51}\text{Cr}$  EDTA was calculated to  $1.08 \times 10^{-6} \text{ cm/s}$  which indicates that the permeability of the capillaries in the myocardium for  $^{51}\text{Cr}$ -EDTA is similar to that of continuous capillaries in other tissues. (B)  $^{51}\text{Cr}$  EDTA ( $3-100 \mu\text{l}$ ) was injected at depth of 5 mm into the myocardium of the left ventricular free wall and the residue curve was recorded. The capillary extraction, as determined by the tissue clearance method, was calculated to values 6 times smaller than determined with the single injection, residue detected method. This unreasonably low extraction was probably due to methodological errors inherent to the tissue clearance technique.

**Key words:** Capillary permeability in canine myocardium.

The filtration-permeability pore model of transcapillary hydrodynamic flow of solvent and diffusive flux of solute was proposed by Pappenheimer and co-workers as a result of a theoretical analysis and the data obtained with the osmotic transient method during filtration (Pappenheimer & Sotoleros 1948) and microgravimetry (Pappenheimer, Rodas & Borrero 1951). From the theories of equivalent pore (Ferry 1936 & Solomon 1968) and restricted diffusion (Fáhræus 1922, Bohlin 1960, Bean 1972, Beck & Schultz 1973, Vermory et al. 1973, Fåhræus & Scherr 1973) it was deduced that circular cylindrical water-filled pores of  $30 \text{ \AA}$  radius or—alternatively—parallel plate channels with a slit width of  $77 \text{ \AA}$  could account for the experimental results obtained in capillaries of the continuous type (Mansueti 1963). The interendothelial slit (Kurovsky

1967, 1968) is generally believed to be the morphological basis for this small pore system. The osmotic transient method is, however, complicated with corrections for the osmotic reflection coefficient (Staverman 1951) and the fact that solvent (water) passes the whole capillary surface area leads to further obstacles as the analysis considers the pores to be the sole transcapillary exchange pathway (Perf 1971, 1973).

A number of studies of capillary diffusive permeability with methods based solely on indicator diffusion principles have consistently provided experimental evidence for a much larger equivalent pore radius of this small pore system (for survey see Paaske 1979). The recently discovered transendothelial channels of fused vesicles (Simionescu, Simionescu & Palade 1975, 1976) appear to be a

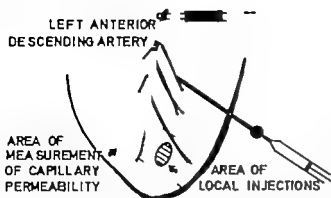


Fig. 1. Schematic illustration showing the preparation of the left descending coronary artery for Cr EDTA and  $^{125}$ I albumin administration.

morphological system that might prove to be the transendothelial pathway for permeation of smaller hydrophilic substances and possibly proteins (Paaske 1976; Paaske & Sejrsen 1977).

The present study was consequently undertaken in order to reexamine the myocardial capillary permeability of the commonly used hydrophilic indicator molecule  $^{51}\text{Cr}$ -ethylenediamine-tetraacetate (Cr EDTA; molecular weight 341.2; molecular radius 4.7 Å; free diffusion coefficient in water at 37°C  $0.70 \times 10^{-5} \text{ cm}^2/\text{s}$ ) with an alternative indicator diffusion technique, i.e. the single injection residue detection method (Sejrsen 1970, 1979). This method has proved well suited to measurement of capillary permeability in other tissues with continuous capillaries (Paaske & Nielsen 1973, 1976; Paaske 1976; Paaske 1977a, b & c; Paaske & Sejrsen 1977; Sejrsen 1979). Further a series of investigations were carried out with a local tissue clearance technique (Lassen & Trap-Jensen 1968a & b) as this method *a priori* seemed useful for the present purposes.

## METHODS

**Animal preparation.** The experiment were performed on 11 mongrel dogs weighing 22 to 27 kg. Anesthesia was introduced by thiobarbital sodium 12.5 mg/kg. The dogs were intubated and artificially ventilated with oxygen and nitrous oxide. I.v. injections of fentanyl (Haldipal) 0.1 mg and pancuronibromidum (Paralun) 2 mg were given when required. A pigtail catheter was introduced through the left femoral artery and the tip placed in the ascending aorta. A Swan-Ganz flow directed thermodilution catheter was inserted through the right external jugular vein with the tip placed in the pulmonary artery. A left thoracotomy was made and the pericardium was incised and sutured to

the thoracic wall. The left anterior descending coronary artery (LAD) was isolated about 2 cm from its origin and a silk snare was placed around the artery to be used for occlusion. A polyethylene catheter was inserted into a small proximal ventricular branch of LAD (Fig. 1) and its tip was positioned just inside LAD. After the operative procedure the dogs were heparinized (400 U/kg).

Throughout the experiments were recorded: Aorta and pulmonary blood pressures, electrocardiogram, body temperature, arterial oxygen saturation, oxygen and carbon dioxide tensions, pH, hematocrit values and cardiac output.

## Experimental procedure

**A. The single injection residue detection experiment.** 20–30  $\mu\text{l}$  Cr EDTA (New England Nuclear 25 mCi/100  $\mu\text{l}$ ) was injected into LAD as a bolus through the polyethylene catheter. The injection lasted about 1 s during which time LAD was occluded proximally by the silk snare. A one inch NaI (TI) scintillation detector located 5 cm above the heart recorded the activity. The detector was connected to a universal preamplifier gamma spectrometer (Meditronic Denmark) with an energy window of acceptance adjusted asymmetrically around 370 keV photo peak of Cr. The sampling integration time was 1 s.

**B. The tissue clearance experiments.** 3–100  $\mu\text{l}$   $^{125}\text{I}$ -Cr EDTA was injected at a depth of 5 mm into the myocardium of the left ventricular wall using a 0.5 mm o.d. needle. The thickness of the ventricular wall of the site of injections was around 11 mm. The activity was recorded as indicated under A using a sampling integration time of 20 s.

**C. Measurement of regional myocardial blood flow** was performed with the local  $^{133}\text{Xe}$  washout technique. 5  $\mu\text{l}$   $^{133}\text{Xe}$  (the Radiochemical Centre, Amersham, England, 10 mCi/ml) in isotonic saline was injected as under B. The gamma radiation of  $^{133}\text{Xe}$  was recorded by a NaI (TI) scintillation detector 5 cm inside the opening of a lead collimator. The detector placed 10 cm above the heart was adjusted to the 81 keV photopeak of  $^{133}\text{Xe}$ . The sampling integration time was 5 s.

**D. Measurement of intracardiac plasma volume** was performed by bolus injection of 30  $\mu\text{l}$   $^{125}\text{I}$  Human serum

Table 1. Blood gas and hemodynamic parameters of 6 dogs measured before and recurrently during each exp.

Results are expressed as mean $\pm$ S.D. n = number of observation			
$P_{aO_2}$ (mmHg)	145–166	(11)	
$P_{aCO_2}$ (mmHg)	32 $\pm$ 9	(11)	
pH	7.39–0.10	(11)	
Hematocrit (per cent)	39–44	(11)	
Heart rate (be $\cdot$ min $^{-1}$ )	142 $\pm$ 43	(11)	
Aortic pressure (mean) (mmHg)	104 $\pm$ 13	(11)	
Pulmonary arterial pressure (mean) (mmHg)	1–4	(11)	
Cardiac output (liter $\cdot$ min $^{-1}$ )	4.7–1	(11)	

Table 2. Capillary extraction  $E$ , and permeability-surface area product  $PS$  (capillary diffusion capacity) of  $^{51}Cr$ -EDTA in the left atricle of the canine myocardium. Plasma flow was calculated from the intravascular transit after curve resolution and compared to plasma flow calculated from the  $^{125}I$ -Xenon washout curve. Hct denotes hematocrit corresponding to each expt.

Plasma flow (ml/100 g min)		Calculated from residue curve of $^{51}Cr$ -EDTA	Calculated from $^{125}I$ -Xenon washout	Hct fraction	PS (ml/100 g min)
Expt.	$E$ ( $^{51}Cr$ -EDTA) %				
1	0.469	69.4	86.0	0.360	39.1
2	0.471	57.1	86.0	0.360	32.5
3	0.295	78.5	86.0	0.360	24.5
4	0.493	63.4	63.4	0.370	38.4
5	0.489	76.8	83.8	0.360	36.0
6	0.271	97.0	83.8	0.360	27.6
7	0.375	71.1	70.0	0.440	29.7
8	0.398	73.3	79.9	0.373	32.3
9	0.088	12.8	9.2	0.030	5.6

solution (Kjeller 0.4 mCi/ml) as described under A. The spectrometer was adjusted around the 364 KeV peak of  $^{51}Cr$ . The sampling integration time was 0.3 s.

E. Microinjection of microcalculation was performed following injection of 30  $\mu$ l  $^{51}Cr$ -EDTA into the cavity of the left atrium, and activity was recorded as indicated under A.

#### Calculations

##### A. The single injection residue detection experiments

The capillary extraction and capillary permeability-surface area product (the capillary diffusion capacity) was calculated as described by Segren (1970, 1979) using the computerized technique of Piazzi & Nisbet (1976). The highest recorded count value which occurred at peak time was taken as relative assessment of the total injected dose. The part of the curve recorded from 20 to 50 s after the bolus injection was extrapolated monoexponentially to peak time using the 'least square' regression procedure.

The numerical value of this function was solved for each 10 s and subtracted from the experimentally recorded curve (corrected for background) to give the intravascular transit function. The value of the monoexponentially extrapolated curve at peak time expressed as fraction of the maximum count value gave capillary extraction,  $E$ . The permeability-surface area product,  $PS$  was calculated as  $PS = f \cdot (E/K) \cdot (1-E)$  (ml/100 g min) (Rankin 1959) where  $f$  is plasma flow and  $K$  is constant for converting ml of plasma to ml of plasma water taking the indicator charge into consideration. For  $^{51}Cr$ -EDTA a  $K$  value of 0.89 was used (Lassen & Trap-Jensen 1968b).  $^{125}I$ -Xenon was calculated as  $R(t)/C(t) \cdot (V(t)/V(t)_{max})$  (Kety 1951), where  $V(t)_{max}$  is the intravascular transit time for the indicator—was calculated as area/height (Zierler 1945) of the extravascular curve obtained by reinterpolation and subtraction. We have the intravascular blood volume of the organ, and Hct is hematocrit value.

B. The lower clearance experiments. Capillary extrac-

tion,  $E$ , was calculated according to Lassen & Trap-Jensen (1968b):  $E = \lambda_{ex} \cdot k_{ex}/(f \cdot \lambda_{ex})$ , where  $\lambda_{ex}$  is the three to blood partition coefficient of  $^{51}Cr$ -EDTA in ml/g (as a reasonable estimate 20 ml/100 g was used), and  $k_{ex}$  (min $^{-1}$ ) is the fractional removal rate of indicator from the labelled area.  $k_{ex}$  was calculated by regression analysing using 'least square' technique on the recorded count figures.

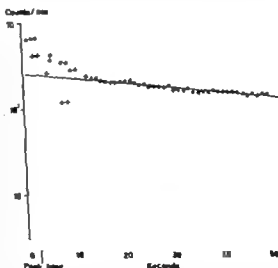


Fig. 2. Curve measured by residue detection after bolus injection of 30  $\mu$ l  $^{51}Cr$ -EDTA. The dots denote the recorded count values corrected for background activity. The monoexponential regression line as calculated by the 'least square' method in the time interval from 20 to 50 s after the bolus injection is shown. Crosses signify the intravascular transit curve obtained by subtraction of the regression line from the original count values (dots).

Table 3 Results obtained from the local tissue clearance expts using different volumes of  $^51\text{Cr}$  EDTA

The fractional removal rate constant  $k_{ex}$  from the labelled area, was calculated by regression analysing using least square technique on the recorded count figures

Expt no	Intramyocardial injected volume ( $\mu\text{l}$ ) of $^51\text{Cr}$ EDTA	$k_{ex}$ (min $^{-1}$ )
8		0.363
9	3	0.183
10	3	0.358
11	3	0.175
12	3	0.42
13	5	0.201
14	5	0.353
15	10	0.317
16	10	0.227
17	100	0.271
$n=10$		$\bar{x}$ 0.261
		$\pm$ S.D. 0.074

C. Calculation of regional myocardial plasma flow  $f(p)_{k_{ex}}$  was made from  $f(p)_{k_{ex}} = k_{ex} \lambda_{50} (1/\text{Hct}) 100$  (ml/100 g min) where  $k_{ex}$  is the slope (min $^{-1}$ ) of the recorded washout curve of  $^{133}\text{Xe}$  and  $\lambda_{50}$  is the tissue to blood partition coefficient of  $^{133}\text{Xe}$  (Kety 1951).  $\lambda_{50}$  was estimated in individual experiments in accordance to measured hematocrit values (Tomnesen & Sejnen 1967).

D. The intravascular plasma volume  $V(iv)_{pl}$  was calculated from the equation  $V(iv)_{pl} = f(p)_{k_{ex}} t(i)_{k_{ex}}$  where  $t(i)_{k_{ex}} = \text{area/height}$  of the recorded curve of the intravascular indicator  $^{125}\text{I}$  albumin.

E. Determination of recirculation was performed by direct recording of activity in the field following bolus injection of  $^51\text{Cr}$  EDTA into the left atrium.

## RESULTS

Variables characterising the physiological state of the preparation during the experiments are shown in Table 1.

### A. The single injection residue detection ex

Table 4 Capillary blood volume  $V(iv)_{blood}$  per 100 g of tissue and the intravascular plasma volume  $V(iv)_{plasma}$  calculated following direct determination of the intravascular mean transit time  $t(i)$  from the area and height of the response curve of  $^{125}\text{I}$ -albumin

$f(p)$  denotes regional plasma flow as determined by  $^{133}\text{Xe}$  washout. Hct denotes hematocrit

Expt. no	$t(i)_{^{125}\text{I-alb}}$ (s.)	$f(p)_{^{133}\text{Xe}}$ (ml/100 g min)	$V(iv)_{plasma}$ (ml/100 g)	$V(iv)_{blood}$ (ml/100 g)	Hct fraction
		93.3	7.8	13.9	0.443
18	5.0	93.3	7.8	13.9	0.443
19	5.0	93.3	7	1.8	0.443
20	4.6		7.6	13.6	

periments. The results are tabulated in Table 3. A representative example of the background corrected externally recorded curve and its resolution is presented in Fig. 2. The bolus injection was performed at time zero. The initial rapid increase in recorded activity reached a maximum value of 6664 counts/s at peak time and was followed by a fast decline. A fairly monoexponential curve was obtained between 20 and 50 s. In this time interval regression analysis was performed by means of the least square method. By extrapolation of the curve section obtained from 20 s to 50 s a value of 7498 counts/s was obtained at peak time. The slope  $k_{ex}$  of the later curve section was  $-0.0136 \text{ s}^{-1}$  with a standard deviation S.D. of 0.0009  $\text{s}^{-1}$ . The capillary extraction  $E$  was  $(7498/6669) = 0.375$ . The mean transit time  $t(iv)$  of the intravascular transit function gave a plasma flow of 71.1 ml/100 g min. PS then became  $PS = -71.1/0.89 \ln(1-0.375) = 29.7 \text{ ml/100 g min}$ .

B. The tissue clearance experiments.  $k_{ex}$  value and injection volumes are shown in Table 3. The average capillary extraction  $E$  was calculated to 0.067—a very low figure as compared to the results obtained with other independent methods—see discussion.

C. Regional myocardial plasma flow was determined with  $^{133}\text{Xe}$  from areas subjected to measurements of capillary permeability is given in Table 2.

D. The intravascular plasma volume appeared from Table 4.

E. Recirculation of  $^51\text{Cr}$  EDTA was found to be 1–3% of the injected dose.

## DISCUSSION

The theoretical basis for the single injection residue detection method has been given by Sejnen

Table 5 Permeability coefficients  $P$  of capillaries in the heart for sucrose,  $^{51}\text{Cr}$  EDTA and inulin from various sources measured with different techniques

PS: indicator diffusion method, OT: osmotic transient method, SI: single injection, residue detection method, TC: tissue clearance method, TU: tissue uptake method

Test substance	$P \cdot 10^6$ (cm/s)	Method	Reference
Sucrose	11.0	TU	Schafer & Johnson 1964
Sucrose	3.9	TU	Vargas & Johnson 1964
Sucrose	0.8	ID	Alvarez & Yudilevich 1969
Sucrose	1.73	ID	Ziegler & Goresky 1971
Sucrose	1.9	OT	Bassingthwaight et al. 1975
Sucrose	1.2	ID	Bassingthwaight et al. 1975
Sucrose	1.22	ID	Laughlin & Dunn 1975
Sucrose	2.2-3.3	ID	Rose & Goresky 1976
Sucrose	1.8	ID	Grabowsky & Bassingthwaight 1976
Sucrose	0.87	ID	Harris et al. 1978
$^{51}\text{Cr}$ EDTA	1.08	SI	Present study
Inulin	0.4	TU	Schafer & Johnson 1964
Inulin	0.54	TU	Vargas & Johnson 1964
Inulin	0.27	ID	Alvarez & Yudilevich 1969
Inulin	0.58	OT	Bassingthwaight et al. 1975
Inulin	0.36	ID	Laughlin & Dunn 1975
Inulin	0.2	ID	Grabowsky & Bassingthwaight 1976
Inulin	0.51	TU	Witzmann et al. 1976

(1978, 1979) and Paaske (1979) and it must be emphasized that only one indicator is necessary in order to obtain the permeability-surface area product (PS). The problem of Taylor diffusion (Lassen & Crome 1970) is eliminated as an intravascular indicator is not necessary. The technique has earlier been applied to a multiple inlet, multiple outlet recirculating system using the technique of bolus injection under stopped flow conditions (cutaneous tissue, Paaske 1976). In a modified form the method was applied earlier to the heart by Parker et al. (1974). These authors simultaneously assessed myocardial blood flow and metabolism using cyclotron produced  $^{15}\text{O}$ -water and  $^{15}\text{O}$ -Hemoglobin and external registration with a positron scintillation camera. The same experimental approach has been performed on the brain by Eichling et al. (1974) and Raichle et al. (1976). Guller et al. (1975) determined myocardial permeability of sodium with the indicator diffusion method. The authors further analyzed the detected residue curves of sodium in accordance with single injection residue detection principles. They found that this method provided variable and inconsistent estimates of capillary permeability and PS. Sodium is a highly permeable substance, and the difference between the rate constant of the intravascular (transmitted) and the extracellular fraction of a bolus will be too small to distinguish reliably between the transfer functions. For

hydrophilic molecules in the order of magnitude as  $^{51}\text{Cr}$  EDTA the present experimental data indicate that such problems are not encountered.

In the present study PS was 32.5 ml/100 g min (Table 2). From generally accepted estimates of capillary surface area,  $S$  of 500 cm<sup>2</sup>/g (Bassingthwaight et al. 1974)  $P$  can be calculated to  $1.08 \times 10^{-6}$  cm/s. This figure is in good agreement with results found earlier with the indicator diffusion method (Table 5 and 6) using sucrose as indicator.  $^{51}\text{Cr}$  EDTA and sucrose have the same permeation and diffusion characteristics for all practical purposes. Capillary permeability of the myocardial capillaries for  $^{51}\text{Cr}$  EDTA is essentially the same as the capillary permeability of  $^{51}\text{Cr}$  EDTA in other organs and tissues with similar capillary morphology (skin, adipose tissue, skeletal muscle, Table 6). The present study consequently supports the hypothesis suggested earlier (Paaske 1976) that capillaries of continuous type exhibit similar permeation characteristics regardless of the tissue in which they are located. This view is supported by the findings of a number of studies (Table 6) that the ratio  $P/D$  ( $D$ =free diffusion coefficient in water) is constant, not only for the various smaller indicators but also from organ to organ.

The ratio of free diffusion coefficients between sucrose or  $^{51}\text{Cr}$  EDTA and inulin is about 3.2. The concept restricted diffusion implies deviation of

Table 3 Results obtained from the local tissue clearance expts using different volumes of  $^{51}\text{Cr}$  EDTA

The fractional removal rate constant  $k_{ex}$  from the labelled area, was calculated by regression analysing using least square technique on the recorded count figures

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C Calculation of regional myocardial plasma flow  $f(\text{pl})_{2x}$  was made from  $f(\text{pl})_{2x} = k_{2x} \lambda_{2x} (1 - \text{Hct}) 100$  (ml/100 g min) where  $k_{2x}$  is the slope (min $^{-1}$ ) of the recorded washout curve of  $^{133}\text{Xe}$  and  $\lambda_{2x}$  is the tissue to blood partition coefficient of  $^{133}\text{Xe}$  (Kety 1951).  $\lambda_{2x}$  was estimated in individual experiments in accordance to measured hematocrit values (Tønnesen & Sejrsen 1967).

D The intravascular plasma volume  $V(\text{iv})_{2x}$  was calculated from the equation:  $V(\text{iv})_{2x} = f(\text{pl})_{2x} t(\text{iv})_{2x}$ , where  $t(\text{iv})_{2x}$  = area/height of the recorded curve of the intravascular indicator  $^{125}\text{I}$ -albumin.

E Determination of recirculation was performed by direct recording of activity in the field following bolus injection of  $^{51}\text{Cr}$  EDTA into the left atrium.

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Expt. no	$t(\text{iv})_{^{125}\text{I}\text{-albumin}}$ (s.)	$f(\text{pl})_{^{133}\text{Xe}}$ (ml/100 g min)	$V(\text{iv})_{\text{plasma}}$ (ml/100 g)	$V(\text{iv})_{\text{blood}}$ (ml/100 g)	Hct fraction
18	5.0	93.3	7.8	13.9	0.443
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## DISCUSSION

The theoretical basis for the single injection residue detection method has been given by Sejrsen

ta could be expected. In some trials very small volumes of indicator (Table 3) were injected but few expts. gave essentially the same results as apts. with larger volumes. These findings might be skewed for if the interstitial space does not behave as a well mixed compartment with respect to the indicator so that concentration gradients exist in the region (Page 1963). Local volume expansion resulting in larger diffusion distances due to mechanical separation of the capillaries might play a pronounced contributory role.

The regional myocardial blood flow as determined from the kinetic analysis of the single injection, residue detection expts. and  $^{133}\text{Xe}$  local clearance technique did not differ significantly ( $P < 0.05$ , paired  $t$ -test). The good correspondence between these two independent blood flow measurements supports the assumption of reasonable accuracy in the  $^{51}\text{Cr}$  EDTA apts. The intravascular plasma volume was found to be 7.6 ml/100 g. This is in good agreement with the values of 7.7 and 7.0 ml/100 g found by Ziegler & Goretsky (1971) and Rose & Goretsky (1976) using sucrose as indicator. An estimate of an intravascular plasma volume of 7.5 ml/100 g seems to be reasonably correct as the intravascular curve of  $^{51}\text{Cr}$  EDTA apts. corresponded very well to the shape of the albumin curves.

In conclusion: (1) the capillary permeability of the myocardium to  $^{51}\text{Cr}$  EDTA ( $P = 1.08 \times 10^{-6} \text{ cm/s}$ ) is similar to the capillary permeability of other tissues to continuous capillaries. This supports the hypothesis that the permeability of continuous capillaries is the same regardless of the tissue in which they are located. (2) The pore size of myocardial capillaries is as yet unsettled but if the theory of identical permeability of continuous capillaries holds true the pores in the myocardium are larger (radius larger than 100 Å) than conventionally assumed from the Pappenheimer-Karnovsky concept of a 37 Å slit width. (3) The single injection, residue detection method provides a useful and simple technique for measurement of capillary permeability in the heart. (4) The tissue clearance method systematically gives estimates of capillary filtration 6 times smaller than expected and cannot be recommended for measurement of capillary permeability in the heart.

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Table 1 Permeability coefficient  $P$  of sucrose  $^{51}\text{Cr}$  EDTA  $^{57}\text{Co}$ -B12 and inulin in organs with continuous capillaries

Abbreviations as under Table 5

Tissue	Indicator	$P \cdot 10^3$ (cm/s)	Method	Reference
Skeletal muscle	Sucrose	0.74	ID	Crone 1963 a & b
	Sucrose	0.84	ID	Trap-Jensen & Lassen 1970
	$^{51}\text{Cr}$ EDTA	0.85	ID	Trap-Jensen & Lassen 1970
	$^{51}\text{Cr}$ EDTA	0.94	ID	Lassen & Trap-Jensen 1970
	$^{51}\text{Cr}$ EDTA	0.89	ID	Trap-Jensen 1970
	$^{51}\text{Cr}$ EDTA	0.78	ID	Trap-Jensen & Lassen 1971
	$^{51}\text{Cr}$ EDTA	1.02	SI	Paaske 1977 c
	EDTA	1.30	ID	Rippe & Grega 1978
	EDTA	1.35	ID	Rippe et al 1978
	$^{51}\text{Cr}$ EDTA	1.16	ID	Sejrsen 1979
	$^{51}\text{Cr}$ EDTA	1.06	SI	Sejrsen 1979
	$^{57}\text{Co}$ -B12	0.59	SI	Paaske 1977 c
	Inulin	0.26	ID	Crone 1963 a & b
	Inulin	0.09	ID	Trap-Jensen & Lassen 1970
Adipose tissue	Inulin	0.12	ID	Trap-Jensen & Lassen 1971
	$^{14}\text{C}$ Inulin	0.20	SI	Paaske & Sejrsen 1977
	$^{51}\text{Cr}$ EDTA	0.95	SI	Paaske & Nielsen 1973 1976
Cutaneous tissue	Sucrose	0.95	ID	Linde et al 1974
	$^{57}\text{Co}$ -B12	0.52	SI	Paaske 1977 b
Cutaneous tissue	$^{51}\text{Cr}$ EDTA	0.88	SI	Paaske 1976
	$^{57}\text{Co}$ -B1	0.56	SI	Paaske 1977 a

the ratio between the permeability coefficients from the ratio between the free diffusion coefficients. The ratio of permeability coefficients between sucrose and inulin (Table 5) using the indicator diffusion method does not provide definite answer to the problem of whether restricted diffusion occurs in the heart for inulin as compared to the smaller indicators. Also other techniques provide contradictory results. The indicator diffusion experiments of Alvarez & Yudilevich (1969) provided evidence for absence of restricted diffusion for inulin as compared to sucrose ( $P_{\text{inulin}}/P_{\text{sucrose}} = 2.9$ ) whereas Laughlin & Diana (1975) found slight restricted diffusion ( $P_{\text{inulin}}/P_{\text{sucrose}} = 3.4$ ) corresponding to pores with a radius of 70–100 Å. Determinations of the osmotic reflection coefficients by Vargas & Johnson (1966) resulted in a pore radius estimate of 35 Å. Using the molecular sieving method Arturson, Groth & Groth (1972) found evidence for a pore radius of about 60 Å. Essentially the same value (radius 55 Å) was found by Bassingthwaite et al (1975) using determinations of the osmotic reflection coefficients. The presence of much larger pores was suggested by Duran & Yudilevich (1978) based on experiments with sodium and glucose. The diffusional water permeability  $P$  is about

$60 \times 10^{-8}$  cm/s (Rose et al 1977) and the hydraulic water permeability  $P_f$  is about  $1.500 \times 10^{-3}$  cm/s (Vargas & Johnson 1964). The ratio  $P_f/P$  is consequently 208 which corresponds to a pore radius estimate of some 50 Å.

Results with the tissue uptake and osmotic transport methods cannot be considered reliable due to serious methodological problems inherent to these techniques. The analysis of the indicator diffusion methods is based on assumptions of ideal Krogh cylinder behaviour of the microvasculature. Rose & Goretsky (1976) found heterogeneity of capillary transit times in the intact dog heart, probably due to a large number of discrete parallel pathways. The influence of heterogeneity on the calculation of  $P$  from the conventional equations is a completely unsolved problem.

The tissue clearance method has provided reasonable results from exercising skeletal muscle (Lassen & Trap-Jensen 1968 a) of the same magnitude as those obtained with the indicator diffusion methods. In the present series the extraction values were clearly underestimated with a factor of about 6 when compared to the other method employed, i.e. the rate constants of the clearance from the interstitium to blood are much smaller

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# Effects of calcium and pH on the mechanical performance of heart muscle in the frog *Rana temporaria* during anoxia and subsequent recovery

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Heart ventricular muscle strips from *Rana temporaria* recover their isometric contractile tension completely within 30 min of reoxygenation following an anoxic period of 60 min in physiological solution at pH 7.6. Corresponding recovery at pH 6.6 is only 43% of pretreatment values. High calcium concentration during the period of anoxia at pH 6.6 and subsequent recovery restores contractile tension to almost pre-anoxic values within 1 h. If however the calcium concentration is increased at the moment of reoxygenation, contractile tension is restored even faster than if high calcium levels were present during anoxia. The loss of contractile tension caused by anoxia is the same at both pH 7.6 and 6.6 with the same calcium concentration.

Comparison of the velocity parameters between high and low calcium experiments always shows greater differences for the contraction velocities than for the corresponding relaxation velocities, independently of the pH. The quotient of these two velocities is used as an index of their relative rate of change. The results are interpreted in terms of calcium and hydrogen ion competition at various subcellular structures and the different influences these ions may have on contractile tension and both contraction and relaxation velocities.

**Key words:** Anoxia, reoxygenation, pH,  $Ca^{++}$ , frog, isometric contractile tension, contraction velocity, relaxation velocity.

The recovery of contractile tension which occurs during reoxygenation after temporary nitrogen induced hypoxia in frog ventricular cardiac muscle has been shown to be more pH-sensitive in the decay of tension, which occurs during the resting anaerobic period (Poupa & Gesser 1975). Competition between calcium and hydrogen ions has been shown to occur at several cellular and molecular levels (see Katz & Hecht 1969, Katz 1970, Williamson et al 1975, St. Louis & Sulakhe 1976, Fabiato & Fabiato 1978) and it is likely that one or more of these cellular structures is intimately involved in the failure of the contractile response of heart muscle during anoxic and ischemic conditions. The functions of the various organelles of cardiac muscle cells during heart failure is dis-

cussed in a detailed review by Džurina, Das & Sharma 1978.

A detailed investigation of the anoxic interactions which occur between and within different cellular and subcellular structures is thus necessary under various conditions, to explain the state of each one of these structures as reoxygenation commences.

While several investigations have been made of the response of mammalian cardiac muscle to hypoxia and reoxygenation (see Tyberg et al 1970, Bing et al 1976) there have been no reports of the interaction of calcium and hydrogen ions during these treatments in either mammals or amphibians.

The present study was therefore performed to examine the role of calcium ions in the contractile response of frog ventricle muscle strips during



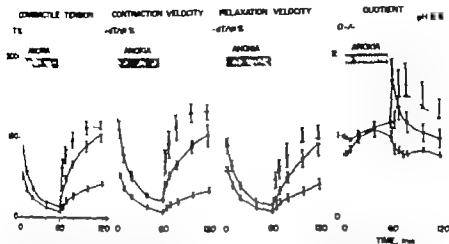


Fig. 1. Mean ( $\pm$  S.E.) of the % values of the isometric contractile tension, contraction and relaxation velocities and the quotient of the velocities, during anoxia and reoxygenation at pH 6.6. Series IV expts. with 1.6 mM Ca (A—A), series V expts. with 7.0 mM Ca (B—B) and series VI expts. with 1.6 mM Ca during anoxia and 7.0 mM Ca during reoxygenation. The results of expts. of series VI are only graphed during reoxygenation as the course during anoxia closely followed the results obtained in the series IV expts.

recovery are of particular interest and will be dealt with separately as follows.

**A. Anoxic decay of contractile tension.** The remaining contractile tension after 60 min of anoxia in experiments run at pH 7.6 is significantly less at 1.6 mM Ca than at 7.0 mM Ca ( $T = 37 \pm 2\%$ ,  $T_0 = 63 \pm 4\%$ ,  $P < 0.01$ ). Also the pre-anoxic values for preparations in low calcium are 100% and in those in high calcium are  $144 \pm 6\%$ . Thus then would indicate that the high calcium muscle has lost more contractile tension during the anoxic period than the low calcium muscle ( $P < 0.01$ ).

At pH 6.6 the contractile tension declines to 71% of its initial value. Anoxia results in a further reduction of the contraction strength which at this pH also gives a resultant contractile tension which is significantly less when the calcium concentration is 1.6 mM than when it is 7.0 mM ( $T = 18 \pm 1\%$ ,  $T_0 = 14 \pm 1\%$ ,  $P < 0.01$ ). As pre-anoxic tension values are  $71 \pm 3\%$  (1.6 mM Ca) and  $115 \pm 8\%$  (7.0 mM Ca) respectively contractile tension loss is again greater at the higher calcium concentration than at the lower concentration ( $P < 0.01$ ).

On the other hand, although significant differences exist between the resultant tension values left after 60 min of anoxia at pH 7.6 and 6.6 ( $P < 0.001$ ) the percentage tension lost during anoxia is identical for experiments using the same calcium con-

centration ( $P > 0.05$ ). During low calcium concentration (1.6 mM) the maximum contraction and relaxation velocities show almost the same percentage changes as does the function of contractile tension at both high (7.6) and low (6.6) pH.

However elevation of calcium concentrations from 1.6 to 7.0 mM causes a dramatic difference between the contraction and relaxation velocities. The contraction velocity at both pH 7.6 and 6.6 is about twice the rate with 7.0 mM calcium as it is with 1.6 mM calcium. The relaxation velocity on the other hand increases only in proportion to the increase in contractile tension (from 100% to 144% at pH 7.6 and from 71% to 115% at pH 6.6) after addition of 7.0 mM Ca (cf. Figs 1 and 2). These differences are also observed during anoxia. The quotient of the contraction and relaxation velocities is  $0.79 \pm 0.02$  at both high and low pH. It increases to  $1.20 \pm 0.07$  at pH 7.6 and to  $1.08 \pm 0.03$  at pH 6.6 after calcium elevation from 1.6 to 7.0 mM. The velocity quotients are stable during anoxia at pH 7.6 but increase towards the end of the nitrogen treatment at pH 6.6.

**B. Reoxygenation recovery of contractile tension.** The ventricular muscle strips exposed to anoxia at normal pH recover upon reoxygenation to pre-anoxic contractile tension values within 20 min independent of the calcium concentration. Cardiac muscle preparations kept at pH 6.6 at low

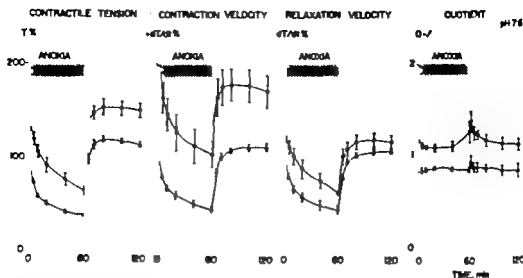


Fig. 1. Mean ( $\pm$  S.E.) of the % values of the isometric contractile tension, contraction- and relaxation velocities and 1 quotient of these velocities during anoxia and reoxygenation at pH 7.6. Series I: expts. with 1.6 mM Ca ( $\Delta$ — $\Delta$ ); series II: expts. with 7.0 mM Ca ( $\bullet$ — $\bullet$ ) are as defined in Table 1.

reoxygenation following 1 h of nitrogen anoxia under both acid and normal physiological conditions.

## MATERIALS AND METHODS

Specimens of the frog *Rana temporaria*, with a body weight of between 10–18 g, were used in this study. They were maintained in fresh water at 8–17°C. The animals were decapitated and despalvated and the heart ventricle dissected ventrally from within the pericardium and separated from the atria. The ventricle was immediately put into a cold (1°C) aerated balanced salt solution of the following composition: Na 16.5, K 5.1, Ca 1.6, Mg 0.9, Cl<sup>-</sup> 132.3, SO<sub>4</sub> 0.9, H<sub>2</sub>PO<sub>4</sub> 2.8, HCO<sub>3</sub> 35.7 mM.

Two ventricular strips with an average cross-sectional area of  $1.15 \pm 0.04$  mm<sup>2</sup> were cut from the apex to the base of each ventricle and mounted in organ baths containing 50 ml of the above balanced salt solution. The solution was kept at 12°C and aerated with a gas mixture of 97% O<sub>2</sub> and 3% CO<sub>2</sub> to give a pH of 7.6.

The upper apical end of the strip was connected to a Grass FT03 isometric transducer. The lower basal end was attached to a fixed muscle holder. The isometric tension and the velocity of its development, expressed as the first derivative of the tension function, were registered on a Grass Polygraph model 7. The muscle strips were paced at a frequency of 12 stimulations/min of 5 ms duration and 200% of threshold voltage. Stimulation was performed with platinum thread electrodes. The muscle strips were stretched and left to beat isometrically at their maximum contractile tension of  $10.536 \pm 0.746$  mN/mm<sup>2</sup> which is described as  $L_{max}$  value.

A number of experimental series were performed varying both the pH and Ca<sup>++</sup> concentration as set out in Table 1. Following a stabilization period of 30–60 min each preparation was aerated with a mixture of 97% N + 3% CO<sub>2</sub> for 60 min to produce anoxia. This was

followed immediately by 60 min of reoxygenation and the 97% O<sub>2</sub> + 3% CO<sub>2</sub> mixture.

The calcium concentration was raised by adding 0.1 ml of a 1.84 M CaCl<sub>2</sub> solution to the organ bath allowing the muscle to stabilize before commencement of anoxia.

The muscle strip exposed to pH 6.6 was also left to stabilize at the new pH before the start of anoxia. Acidification was achieved by slowly adding approximately 6 ml of 12 M HCl to the bath while continuously reading pH. Contractile tension and both contraction (+dT/dt) and relaxation (−dT/dt) velocities were expressed in % of the stabilized  $L_{max}$  value. The Student's *t*-test was used to determine significant differences.

## RESULTS

The results for each of the different experimental conditions are graphically presented in Figs 1 and 2. The decay of contractile tension during anoxia and the recovery of contractile tension upon

Table 1. Ca<sup>++</sup> concentration and pH in different experimental series.

Experimental series	No of expts	Anoxia		Recovery	
		pH	Ca (mM)	pH	Ca (mM)
I (control)	9	7.6	1.6	7.6	1.6
II	6	7.6	7.0	7.6	7.0
III	3	7.6	1.6	7.6	7.0
IV	9	6.6	1.6	6.6	1.6
V	8	6.6	7.0	6.6	7.0
VI	6	6.6	1.6	6.6	7.0

But even, it is not possible to know the extent to which the intra-cellular pH is depressed. According to Ellis & Thomas (1976) the change in intracellular pH of sheep heart Purkinje fibre following a reduction of the external bicarbonate concentration was small and slow. If this is also true for these frog atrial muscle cells, the fact that the contractile tension recovers more slowly in muscle strips held in 7.0 mM calcium during anoxia than in preparations supplied with extra calcium only during reoxygenation, implies that the combination of acid conditions and high calcium concentration during anoxia renders the sarcolemma less permeable to calcium ions than at low pH and normal calcium concentration.

Another explanation might be, however, that the high energy phosphate reserves are reduced much more considerably as a result of the stronger contractions occurring during the anoxic period with high calcium than they are at lower calcium levels. This reflection would also increase the glycolytic rate, and hence increase the intracellular hydrogen ion concentration. However, it must be noted that the intracellular acidification and possibly reduced energy content cannot have caused impairment of the contractile mechanisms to any large extent as contractile tension of high calcium treated preparations after one hour of anoxia is still significantly higher than the contractile tension seen in the low calcium preparations.

Furthermore, if there is also an intracellular decrease in pH resulting from an increase in extracellular hydrogen ion concentration, the possible interactions between hydrogen and calcium ions at subcellular structures may strongly disturb contractility. The sarcoplasmic reticulum sequesters calcium more actively in an acid environment, provided there is enough energy (Nakamura & Schwartz 1972, Schwartz et al 1973). Mitochondria are able to accumulate calcium ions not only at the expense of ATP formation, but also in the absence of other respiratory or ATPase activity by a diffusion potential which is generated by a hydrogen ion gradient (Schwartz et al 1970, Lehninger 1974). The myofibrils also exhibit competitive interaction between calcium and hydrogen ions, as submaximal cross development is decreased by lowering the pH (Schäfer 1967, Fabiato & Fabiato 1978).

Thus, these subcellular structures can accumulate and/or release calcium ions in different ways, depending on their environment. The faster

tension recovery in muscle strips treated with high calcium only during reoxygenation indicates that one or more of these intracellular organelles have modified their mechanisms during anoxic high calcium treatment in a way that renders the myocardial cell less sensitive to calcium ions in spite of identical extracellular conditions.

It is clear however from this study that the weak myocardial recovery of contractile tension upon reoxygenation following one hour of anoxia at pH 6.6 can be significantly increased by addition of calcium ions even in acid conditions. Further work will be necessary to elucidate the exact mechanism by which this occurs.

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Table 2 Means ( $\pm$  S.E.) of % values of contractile tension contraction and relaxation velocity after 30 min of reoxygenation in expts. of series V and VI

Experimental series	T	+dT/dt	-dT/dt
V	79 $\pm$ 6	78 $\pm$ 7	61 $\pm$ 8
VI	108 $\pm$ 9	143 $\pm$ 13	86 $\pm$ 17

calcium concentration do not return to preanoxic tension values when reoxygenated. After 60 min recovery only 43 $\pm$ 3% of the initial contractile tension remains. With 7.0 mM calcium ions recovery is almost complete after 1 h in spite of the low pH.

Accordingly there is a more pronounced difference in recovery capability between the high and low calcium muscle preparations at pH 6.6 than at pH 7.6. Even during reoxygenation the changes in contraction and relaxation velocities resemble the changes in contractile tension in experiments with low calcium concentration. With 7.0 mM calcium the divergent response of contraction velocity mentioned above is maintained.

Although it is difficult to observe any differences between the contraction and relaxation velocity during recovery the velocity quotient does however indicate a more rapid increase in the contraction velocity than in the relaxation velocity during the first 3 min of reoxygenation. Within these first minutes of recovery the quotients of the velocities show peak values which are higher at the lower pH with high calcium. The quotient peak is more prolonged when 7.0 mM calcium is added at commencement of reoxygenation at pH 6.6 (Exp. series VI). This group of muscle strips recover from acidified anoxia significantly faster ( $P < 0.05$ ) than preparations exposed to 7.0 mM  $\text{Ca}^{2+}$  even during the anoxic period (Table 2).

No such difference in recovery-rate occurs in the corresponding experiments at pH 7.6. After 60 min reoxygenation however there is no significant difference between the % values of recorded parameters in the experimental series V and VI. In all experiments with the same calcium content during both anoxia and reoxygenation the quotient values return to preanoxic levels within 1 h.

## DISCUSSION

The present investigation has shown responses to hypoxia and reoxygenation for frog cardiac muscle

which are similar to those shown for mammalian cardiac muscle by Tyberg et al. (1970) and Bagge et al. (1976). However our results provide further insight into the possible mechanisms involved in these responses.

Acid conditions have been shown to suppress contractile tension development in frog ventricular isometric muscle strips during recovery from period of anoxia (Poupa & Gesser 1975). Pool et al. (1966) showed that the reduction in high energy stores during hypoxia is not the primary limit to contractile force development and conclude that depression of myocardial function was initiated before a decrease in ATP content. The present work also clearly shows that contractile tension suppression under acid conditions cannot simply be due to a lack of metabolic energy, as an increase in calcium concentration can induce rapid recovery during reoxygenation. The poor recovery under acid conditions following anoxia must therefore be explained by other interactions involving calcium ions. As calcium ions activate myofilaments (Kat 1970) and as the negative effect of hydrogen ions on tension development can partly be reversed by increasing the extracellular calcium ion concentration it seems likely that the depressant action of hydrogen ions on the recovery of contractile tension is caused by interactions with calcium binding and/or releasing structures in the myocardial cell.

The first barrier to calcium ion penetration is the sarcolemma which in cardiac muscle cells is a very important source of calcium ions. Unlike skeletal muscle cells the myofilaments of cardiac muscle cells cannot be completely activated by the release of intracellular calcium alone. Thus the myocardium is highly dependent on an adequate calcium influx through the sarcolemma which either by itself or by its induction of the release of calcium from the sarcoplasmic reticulum (Fabiato & Fabiato 1978) can bring about filament sliding. It has also been reported by Kohlhardt et al. (1976) that the slow inward current observed during the action potential is diminished by a decrease in pH and both St. Louis & Sulakhe (1976) and Williamson et al. (1975) have shown that the calcium binding affinity of the sarcolemma is inhibited at pH 8.8 compared to pH 7.4.

Hence as the cardiac muscle cells in the present expts. are exposed to hydrogen ions in the bathing solution the transport of calcium across the sarcolemma is likely to be suppressed.

# Mechanism of action of pentobarbital on the contractile system of isolated frog muscle fibres

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The effects of pentobarbital-sodium were studied on single muscle fibres of frog skeletal muscle. A concentration of 0.5 mM pentobarbital potentiated the twitch response without significantly affecting the tetanus amplitude. The increase in twitch amplitude was accompanied by marked increase in the rate of force development. The relaxation phase of both twitch and tetanus was prolonged. These mechanical changes were associated with an increased duration of the action potential. Pentobarbital also increased the amplitude and the rate of force development during the twitch after suppression by dantrolene. The S-shaped curve relating peak contracture tension and log caffeine concentration was shifted to the left by pentobarbital. It is suggested that pentobarbital affects the contractile activity of the muscle fibre: (1) by increasing the rate of release of calcium from its storage sites, (2) by prolonging the duration of the action potential and (3) by inhibiting calcium re-sequestration by the sarcoplasmic reticulum (SR).

**Key words.** Pentobarbital skeletal muscle, single muscle fibre, excitation-contraction coupling, twitch potentiation, action potential.

Anesthetics are known to have direct effect on muscle contraction. Pentobarbital for example has been shown to potentiate the twitch response of *Xenopus* as well as amphibian muscles (Quilism 1955a, Gjone 1956, Foulks et al. 1973, Holmberg & Naflek 1979). The stimulatory effect of barbiturates on the twitch response of muscle has been attributed to an increased duration of the action potential (Quilism 1955b, Gjone 1956, Foulks et al. 1973), although it has been suggested that they may potentiate the twitch by interfering with the relaxation mechanism (Gjone 1956). This latter assumption is supported by the findings that barbiturates inhibit calcium uptake by the sarcoplasmic reticulum (SR) isolated from heart (Briggs et al. 1966).

The present study was undertaken as an attempt to obtain further information concerning the mode of action of barbiturates on skeletal muscle. Evidence will be presented to show that pentobarbital greatly potentiates the twitch response and also prolongs relaxation in isolated frog muscle fibres. The twitch potentiation may be attributed to an increased duration of the action potential. It is

suggested that the slowing of the relaxation is due to an inhibitory effect of pentobarbital on calcium re-sequestration by the SR.

## METHODS

Effects of pentobarbital were studied on muscle fibres isolated from the ventral head of the semitendinosus muscle of *Rana temporaria*. The methods used for mounting, stimulation, temperature control and for production of caffeine contracture are described elsewhere (Khan & Edman 1979).

**Recording of membrane potential.** Resting and action membrane potentials were recorded in the presence and absence of pentobarbital (0.5 mM) using conventional glass capillary electrodes (Khan & Edman 1979).

In these experiments the bath temperature varied between 2.8 and 3 °C but was maintained constant to within  $\pm 0.2^\circ\text{C}$  throughout any particular experiment, even during exchange of solutions.

**Solutions.** A Ringer solution of the following composition was used (mM): NaCl 115.5, KCl 2.0,  $\text{CaCl}_2$  1.8, Na-phosphate buffer 2.0, pH 7.0.

Pentobarbital-sodium (0.85-1.0 mM), barbitone-sodium (1.0 mM) and dantrolene-sodium (9.0  $\mu\text{M}$ ) were dissolved in Ringer solution and care was taken to adjust the pH to 6.8-7.5.

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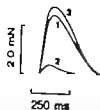


Fig. 2 Effects of pentobarbital on isometric twitch of frog muscle fibre pretreated with dantrolene. (1) Control, normal Ringer solution. (2) In the presence of dantrolene ( $9.0 \mu\text{M}$ ) (3) In the presence of dantrolene ( $9.0 \mu\text{M}$ ) and pentobarbital ( $0.2 \text{ mM}$ ).

lateral, the tetanic force however was slightly depressed. Similar to the situation in the twitch response, the time to half decay of tension after the last stimulus of the tetanus response was also prolonged.

The effects produced by pentobarbital were to a large extent reversible by repeated washing of the fibre with Ringer solution. An increase of the pentobarbital to  $1.0 \text{ mM}$  rendered the fibre inexcitable to electrical stimulation.

Dantrolene, a powerful muscle relaxant, is known to suppress the twitch response in a single muscle fibre (Halpern & Desmedt 1974; Desmedt & Roussot 1977; Edman 1979). The effects of pentobarbital were also studied in the presence of dantrolene ( $9.0 \mu\text{M}$ ). As illustrated in Fig. 2, pentobarbital ( $0.2 \text{ mM}$ ) potentiated the dantrolene suppressed twitch with a marked increase in the rate of force development.

Contractions were induced by different concentrations of caffeine in the presence and absence of pentobarbital ( $0.5 \text{ mM}$ ). The peak tension developed as plotted against the log concentration of caffeine. As illustrated in Fig. 3 pentobarbital shifted the S-shaped curve relating contracture tension and log caffeine concentration to the left.

Other barbiturates like barbitalone with low lipid solubility seem to have little effect on the contractile activity of the muscle fibre. As illustrated in Fig. 4 barbitalone in a concentration of  $1.0 \text{ mM}$  had almost no effect on the twitch response.

**Effect of pentobarbital on resting and action membrane potential**

Pentobarbital did not cause any significant change of the resting membrane potential. The overshoot and the maximum rate of rise of the action potential were also not changed significantly. Pentobarbital,

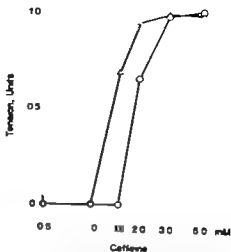


Fig. 3 Effects of pentobarbital on the relation between peak contracture tension and caffeine concentration (log scale) in single muscle fibres. O in the absence of pentobarbital.  $\Delta$ , in the presence of pentobarbital ( $0.5 \text{ mM}$ ). The maximum tension developed by  $5 \text{ mM}$  caffeine in the absence of pentobarbital was taken as  $1.0$  unit. Each point represents mean value of 3 different experiments.

however reduced the rate of repolarization, causing an increased duration of the action potential (Fig. 5 and Table 1). These results are consistent with earlier findings in studies on whole muscle preparations (Quilliam 1955b; Thealeff 1956; Foulka et al. 1973).

## DISCUSSION

The results presented here show that pentobarbital potentiates the isometric twitch amplitude of the single muscle fibre and increases the duration of the action potential. These findings are in agreement with results obtained previously in studies of mammalian and amphibian whole muscles (Quilliam

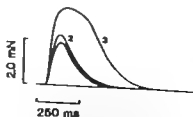


Fig. 4 Comparison of pentobarbital and barbitalone effects on isometric twitch of single muscle fibres. (1) Control, normal Ringer solution. (2) In the presence of  $1.0 \text{ mM}$  barbitalone. (3) In the presence of  $0.5 \text{ mM}$  pentobarbital.

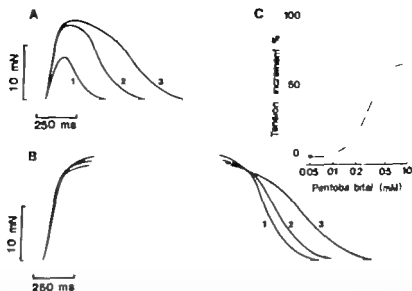


Fig. 1 Effects of pentobarbital-sodium on isometric twitch (A) and tetanus (B) in frog single muscle fibre: (1) Control normal Ringer solution; (2) 0.5 mM pentobarbital; (3) 1.0 mM pentobarbital. Stimulus markers below the base line. Concentration-response curve (C). Ordinate: Increase in twitch tension in per cent of control (no pentobarbital). Abcissa: pentobarbital concentration (log scale). Each point represents mean value of 3 expts.

All chemicals used were of analytical grade and were dissolved in double distilled water.

Pentobarbital-sodium and barbitone-sodium were obtained from B.D.H. Chemicals, Poole, England. Dantrolene-sodium was from Eaton Chemicals, Norwich, N.Y., USA.

## RESULTS

The effects of pentobarbital were studied on twitch and tetanus responses of single muscle fibres. It can be seen in Fig. 1A that pentobarbital (0.5 and 1.0 mM) greatly potentiated the twitch amplitude and increased the rate of development of tension. According to the concentration response curve (Fig.

1C) maximum potentiation of the twitch was obtained with 1.0 mM pentobarbital. The increase in amplitude of the twitch was accompanied by a slight increase in the time to peak tension and a marked prolongation of the relaxation phase (Fig. 1A and Table 1). The magnitude of the twitch potentiation was inversely related to the twitch/tetanus ratio recorded in normal Ringer solution. When the effect of pentobarbital was maximal (1.0 mM) the amplitude of the twitch reached about 90% of the tetanic force.

Pentobarbital (0.5 mM) caused no significant change in the amplitude of the tetanus response (Fig. 1B). At a bath concentration of 1.0 mM peno-

Table 1 Effects of pentobarbital (0.5 mM) on the twitch parameters in frog single muscle fibres. Each value represents the mean of 4-6 twitch responses.

Expt. no.	Normal Ringer		0.5 mM pentobarbital		$T_p/T$	$R_p/R_c$
	Time for 1/2 peak tension (ms) $T_c$	Time for 1/2 relaxation (ms) $R_c$	Time for 1/2 peak tension (ms) $T$	Time for 1/2 relaxation (ms) $R_p$		
1	61	96	79	219	1.3	2.3
2	53	114	70	324	1.3	2.8
3	61	88	88	167	1.4	1.9
Grand mean	58	99	79	236	1.3	2.3

doi: 60-739) (Balzer & Khan 1975). Tada et al. (1978) previous studies suggest that drugs like re-typer, chlorpromazine and prenylamine exert an inhibitory action on SR calcium uptake by binding to the unsaturated fatty acids of the SR lipid (Balzer et al. 1968). A similar binding of pentobarbital in the lipid phase of the SR might cause a slowing of calcium reuptake and hence be responsible for the prolongation of the relaxation phase.

In conclusion pentobarbital seems to influence the contractile activity of the muscle fibre in 3 different ways:

1. By enhancing the rate of release of calcium from the storage sites, pentobarbital causes an increased rate of force development and an increased amplitude of the isometric twitch.

2. By prolonging the duration of the action potential the release of calcium occurs over a longer time. As a result, it adds to factor 1 causing an increased amplitude of the isometric twitch.

3. Pentobarbital probably inhibits the calcium re-uptake by the SR leading to a reduced rate of relaxation of the twitch and tetanus response.

This work was supported by grants to Professor K. A. P. Khan from the Swedish Medical Research Council (Project No. 147, 184) and from The Muscular Dystrophy Association of America and personal grants to the author from The Medical Faculty, University of Lund.

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Table 7 Effects of pentobarbital (0.5 mM) on resting and action membrane potentials of frog muscle fibres (mean  $\pm$  S.E.)

Before recordings were made the fibre bundles were exposed to pentobarbital for 15–20 min. Number of fibres for each measurement given within brackets. Student's *t*-test  $P < 0.001$

	Action potential				
	Resting potential (mV)	Overshoot (mV)	Maximum rate of rise (V/s)	Maximum rate of decay (V/s)	Duration at $-25$ mV level (ms)
Control	$86.9 \pm 7$ (12)	$40.0 \pm 8$ (12)	$64.2 \pm 5.1$ (12)	$77.7 \pm 1.0$ (12)	$4.8 \pm 0$ (12)
Pentobarbital	$84.4 \pm 3$ (17)	$37.4 \pm 2.9$ (12)	$63.2 \pm 6.4$ (11)	$18.5 \pm 1.5$ (12)	$6.2 \pm 0.4$ (12)

1955b Gjone 1956 Thesleff 1956 Foulks et al 1973). As the action potential may be assumed to govern the release of activator calcium (Sandow et al 1965 Edman et al 1966 Taylor et al 1977) its prolongation would cause an increased release of calcium from the storage sites. However if this were the only factor causing twitch potentiation then there would be no change of the initial rate of rise of tension. The fact that the rate of force development is markedly steeper in the presence of pentobarbital suggests that the rate of calcium release is increased not merely the duration of the release. Further evidence in support of this idea is provided by the fact that pentobarbital counteracts the effects of dantrolene. The latter agent inhibits the depolarization induced release of calcium from the storage sites (Hainaut & Desmedt 1974 Desmedt & Hainaut 1977) leading to a marked decrease of both the rate of rise and amplitude of the twitch (Hainaut & Desmedt 1974 Desmedt &

Hainaut 1977 Edman 1979). Pentobarbital restores the speed of force development in the presence of dantrolene suggesting that the rate of release of calcium was indeed increased.

On the basis of studies carried out on muscle from the rat diaphragm Gjone (1956) suggested that barbiturates affect muscle contraction by inhibiting the relaxation process. It is now well established that relaxation in skeletal muscle is achieved when calcium is resequestrated by the sarcoplasmic reticulum (SR). An inhibitory action of pentobarbital on the calcium pump would therefore prolong the relaxation phase of the twitch as well as of the tetanus response as was actually observed in the present experiments.

If pentobarbital inhibits the reuptake of calcium by SR this would tend to raise the calcium concentration in the myofibrillar space during calcium release by caffeine. As caffeine is assumed to induce contractions by enhancing the release of calcium from the storage sites (Endo 1975) this would be expected to lower the concentration of caffeine required to induce contracture. In accordance with this prediction pentobarbital was found to shift the S-shaped curve relating peak contracture tension and log caffeine concentration to the left. This suggests that pentobarbital like diethylstilboestrol may have an inhibitory action on the SR calcium resequstration. Such an effect on calcium pump of the SR may also contribute to the twitch potentiation as suggested previously (Khan 1979).

It appears likely that pentobarbital being very lipid soluble readily penetrates the cell membrane to have access to intracellular structures like SR. The SR membranes are rich in phospholipid contents and have a high percentage of phosphatidyl-

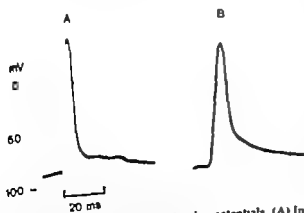


Fig. 5 Intracellular recordings of action potentials (A) in normal Ringer solution (B) in the presence of 0.5 mM pentobarbital. The recordings in A and B are from different muscle fibres and slightly retouched for greater clarity.

choline (60-73%) (Balzer & Khan 1975; Tada et al 1976). Previous studies suggest that drugs like verapamil, chlorpromazine and procainamide exert their inhibitory action on SR calcium uptake by binding to the unsaturated fatty acids of the SR lipid phase (Balzer et al 1968). A similar binding of pentobarbital to the lipid phase of the SR might cause a slowing of calcium reuptake and hence be responsible for the prolongation of the relaxation phase.

In conclusion pentobarbital seems to influence the contractile activity of the muscle fibre in 3 different ways:

1. By enhancing the rate of release of calcium from the storage sites, pentobarbital causes an increased rate of force development and an increased amplitude of the isometric twitch.

2. By prolonging the duration of the action potential the release of calcium occurs over a longer time. This effect, in addition to factor 1, causes an increased amplitude of the isometric twitch.

3. Pentobarbital probably inhibits the calcium reuptake by the SR leading to a reduced rate of relaxation of the twitch and tetanus response.

The work was supported by grants to Professor K. A. P. Ekman from the Swedish Medical Research Council (Project No. 14X 184) and from The Muscular Dystrophy Association of America and personal grants to the author from The Medical Faculty, University of Lund.

The technical assistance of Britta Kronborg and Eva Ekman are gratefully acknowledged.

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- THESLEFF, S. 1956. The effects of anaesthetic agents on skeletal muscle membrane. *Acta Physiol Scand* 37 333-349.





## Effect of isotonic volume expansion on glomerular filtration rate and renal hemodynamics in the developing rat kidney

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ELINDER, G., APERIA, A., HERIN, P. & KÄLLSKOG, Ö. Effect of volume expansion on glomerular filtration rate and renal hemodynamics in the developing rat kidney. *Acta Physiol Scand* 1980, 108, 411-417. Received 20 July 1979. ISSN 0001-6772. Department of Pediatrics, Karolinska Institute, St. Görans Children's Hospital, Stockholm and Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden.

Young rats (20-34 days) and adult rats (40-45 days) were studied during hydropenic and volume expansion with regard to glomerular filtration rate (GFR) and the determinants of GFR. During hydropenia, GFR and renal blood flow (RBF) were significantly lower in younger than in adult rats both in absolute terms and when related to bodyweight. Equivalent degrees of volume expansion (6% of b.wt.) resulted in much more pronounced increases in GFR and RBF in younger than in older rats. This suggests that the high renal vascular resistance in hydropenic young rats is primarily due to vasoconstriction. The relationship between the filtration rate of superficial nephrons and the total GFR was the same in hydropenic and volume expanded rats in both age groups. The tubular stop flow pressure (the calculated hydrostatic glomerular capillary pressure and ultrafiltration pressure in the efferent part of the glomerular capillaries) was slightly lower in hydropenic young rats than in hydropenic adult rats. The pressures did not rise after volume expansion. It is concluded that the marked increase in GFR in volume expanded young rats is mainly due to increased renal plasma flow.

**Key words:** Renal functional development, intrarenal vascular resistance, renal blood flow, glomerular filtration pressure, tubular pressure, isotonic volume expansion.

During early stages of postnatal development it is well documented in man (Aperia et al. 1972), dog (Odumson & Reuter 1974), lamb (Aperia et al. 1973) and rat (Benzels & Solomon 1973) that the natriuretic response to volume expansion is blunted. This effect has often been ascribed to the low glomerular filtration rate (GFR) of the neonate. It is somewhat surprising, however, that the GFR is considerably higher in well hydrated neonatal infants than in hydropenic infants (Broberger 1973, Brodeur et al. 1972, Leake et al. 1976). This indicates that infants have a reduced natriuretic response to volume expansion despite a large increase in the filtered load of Na. If this is correct, volume expansion in infancy should result in an increased GFR, increased filtered load of Na and increased tubular reabsorp-

tion of Na. This would probably lead to an increased energy demand and therefore an increased need for oxygen by the kidney.

The aim of the present study was to better elucidate the effects of isotonic volume expansion on GFR and determinants of GFR of the infant rat kidney. Hydropenic and volume expanded 20 to 24-day-old rats were compared to hydropenic and volume expanded 40 to 45-day-old rats with regard to renal blood flow, GFR, single nephron filtration rate and glomerular capillary pressure.

In the adult kidney isotonic volume expansion causes an increase in renal blood flow and GFR (Barrai et al. 1973, Brenner et al. 1972, Stein et al. 1972, Wallin et al. 1971, Wallin et al. 1972). The increase in GFR appears to be related to the amount

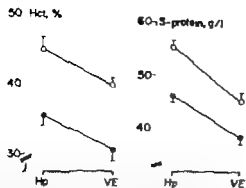


Fig. 1 Hematocrit (Hct) and serum protein concentration (G<sub>0.3</sub>) in hydroponic and 6% volume expanded 20 to 24-day-old rats (closed circles) and 40 to 45-day-old rats (open circles).

of fluid given (Wallin et al 1972). For this reason special attention was paid to the relationship between the degree of volume expansion on the one hand and renal blood flow and glomerular filtration rate on the other.

## MATERIAL AND METHODS

Male Sprague Dawley rats aged 20–4 days (mean  $\pm$  S.E.  $23.3 \pm 0.2$ ) and 40–45 days (mean  $\pm$  S.E.  $41.9 \pm 0.4$ ) delivered from Antikimex were studied during hydropenia and following volume expansion. The body weight averaged  $63.1 \pm 1.4$  (mean  $\pm$  S.E.) in the younger group and  $178.9 \pm 7.5$  (mean  $\pm$  S.E.) in the older group. During this age period there is a linear relationship between body weight, and age and between kidney weight and age (Aperia & Hern 1975). The rats were weaned at 17 days

of age and prior to the study all rats were given an ordinary laboratory diet and water ad libitum.

Most studies have been performed during hydropenia and after isotonic volume expansion corresponding to 6% of body weight (VE<sub>6%</sub>). Hence the rats were divided into four groups:

1a 20 to 24-day-old rats studied during hydropenia

1b 20 to 44-day-old rats studied during volume expansion (6%)

2a 40 to 45-day-old rats studied during hydropenia

2b 40 to 44-day-old rats studied during volume expansion (6%)

The following parameters of renal function were determined: renal blood flow (RBF), glomerular filtration rate (GFR), single nephron glomerular filtration rate (SNGFR), tubular free flow pressure (P<sub>FF</sub>), tubular flow pressure (SFP) and pressure in the efferent arteriole ("welling point") (P<sub>1</sub>). The condition of young rats often deteriorates if the duration of the experiment exceeds 4 h. The studies were therefore performed either during hydropenia or volume expansion. In addition different rats were used for RBF and micropuncture studies. The preparation of the animals, however, was the same in most respects in all of the hydropenic and volume expansion studies.

For this reason animals in each group had the same average arterial pressure and hematocrit regardless of whether they were used for renal blood flow or for micropuncture studies. The hematocrit was lower in the younger than in older rats but it was higher in the 6% expanded older rats than in the hydropenic young rats. Consequently another group of 40 to 45-day-old rats (2 rats included) These rats were volume expanded until their hematocrit levels fell within the same range as that observed in the younger rats. Their isotonic volume expansion corresponded to 10% of body weight (VE<sub>10%</sub>). If

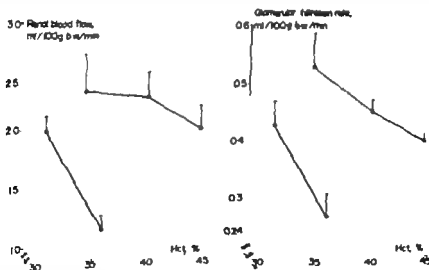


Fig. 2 Relationship between hematocrit (Hct) and renal blood flow (RBF) and between hematocrit and glomerular filtration rate (GFR) in 20 to 44-day-old and 40 to 45-day-old rats. Symbols as in Fig. 1.

Table 1. Total renal blood flow (RBF) and glomerular filtration rate (GFR) in hydropenic and volume expanded 20 to 24 and 40 to 44-day-old rats

Values are mean  $\pm$  S.E. n = number of rats per group (in parentheses)

	RBF (ml/min)	RBF (ml/100 g h wt./min)	GFR (ml/min)	GFR (ml/100 g h wt./min)
Hyd 20-24 d (6)	0.72 $\pm$ 0.09	1.13 $\pm$ 0.14	0.17 $\pm$ 0.03	0.27 $\pm$ 0.04
Hyd 40-44 d (8)	1.30 $\pm$ 0.01	1.99 $\pm$ 0.11	0.77 $\pm$ 0.03	0.43 $\pm$ 0.04
Vol 20-24 d (6)	3.42 $\pm$ 0.34	2.01 $\pm$ 0.1	0.73 $\pm$ 0.05	0.41 $\pm$ 0.03
Vol 40-44 d (7)	3.95 $\pm$ 0.33	2.29 $\pm$ 0.19	0.81 $\pm$ 0.04	0.45 $\pm$ 0.02
Vol 40-44 d (8)	4.14 $\pm$ 0.45	2.2 $\pm$ 0.26	0.94 $\pm$ 0.08	0.53 $\pm$ 0.05
Hyd 20-24 d	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Hyd 40-44 d	$P < 0.001$	$P < 0.002$	$P < 0.001$	$P < 0.02$
Vol 20-24 d	$P < 0.001$	n.s.	$P < 0.001$	n.s.
Vol 40-44 d	$P < 0.02$	$P < 0.02$	$P < 0.001$	$P < 0.001$

only the total RBF and the total GFR were measured.

**Microvascular procedures.** The rats were prepared for experiments in the same manner described elsewhere in this laboratory (Aperia & Hern 1975). Microvascular procedures as performed under Leitz stereo microscope at magnification of 64 $\times$  and with the aid of one of two Leitz micro-manipulators. The SNGFR was determined early segments of the proximal tubule with a laser Doppler capillary (Schneeweis et al. 1969). The diameter varied between 6 and 9  $\mu$ m depending on the size of the animal. The tubular fluid was collected spontaneously proximal to blocking out drop for time interval ranging between 60 and 200. The longer sampling interval was used in the younger animals. Approximately 4

collections were made in each animal and the SNGFR was calculated as the mean of all values obtained. The GFR was obtained simultaneously by collecting urine from ureter via polyethylene catheter that was advanced almost to the entrance of the pelvis. In some experiments it was suspected that the catheter caused partial obstruction. In these cases the catheter was immediately removed to allow free flow of urine. In these rats only SNGFR could be estimated. Arterial blood samples were taken from the femoral artery catheter. The concentration of urea in blood and in urine samples was analyzed with the azoture method (Häger et al. 1958). The hydrostatic pressures were measured with servo-controlled counter pressure device based on the principles outlined by Wederikheim et al. (1964) and modified by Ingber et al. (1970). In all animals each pressure parameter was determined at least 4 times during hydropenia and during volume expansion. The average value for each animal was then calculated.

**Determination of oncotic pressure.** Plasma protein concentration ( $C_p$ ) was determined from arterial blood samples with the Buret method (Kingsley 1959). The albumin-globulin quotient was obtained by plasma electrophoresis. Since the quotient averaged 1.1 it was thought to be justified to use the equation given by Landis & Pappenheimer (1963) for calculation of the oncotic pressure in arterial blood ( $2.1C_p + 0.16C_p + 0.009C_p^2$ ; Eq. 4).

**Determination of blood flow.** Microspheres ( $^{51}Cr$  3M Co. St. Paul, Minnesota) measuring 15  $\mu$ m in diameter mean  $\pm$  S.D. ( $14.6 \pm 0.8$ ) were used to quantify the renal blood flow. The method used in our laboratory earlier has been described elsewhere (Aperia & Hern 1975). Absolute values for blood flow were obtained by the reference

100-mm Hg/min ml/100g bw

80-

60-

Hyd Vol

Fig. 1. Total renal vascular resistance (RVR) in hydropenic and 6% volume expanded 20 to 24 and 40 to 44-day-old rats. Symbols as in Fig. 1.

Table 2 Nephron filtration rate, intrarenal pressure and arterial and venous blood pressures in hydronephrotic and volume expanded 20 to 24 and 40 to 45-day-old rats

	SNRFR (nl/min/ 100 g b wt)	P <sub>oc</sub> (mmHg)	SFP (mmHg)	$\Pi_{AA}$ (mmHg)	P <sub>r</sub> (mmHg)	P (mmHg)
1a 20-24 d (Hp)	5.99±0.71 (n=8)	45.0±0.9 (n=5)	31.3±0.5 (n=8)	14.1±0.5 (n=5)	14.5±0.8 (n=8)	13.7±1.3 (n=6)
1b 20-24 d (VE <sub>45d</sub> )	11.90±1.18 (n=8)	38.6±4.9 (n=5)	28.3±7.0 (n=5)	10.3±1.3 (n=5)	14.6±3.4 (n=9)	12.7±4.7 (n=5)
2a 40-45 d (Hp)	10.5±0.54 (n=8)	51.8±1.7 (n=5)	33.3±1.6 (n=6)	18.1±0.8 (n=5)	14.4±0.6 (n=6)	12.9±0.3 (n=6)
2b 40-45 d (VE <sub>45d</sub> )	12.79±0.40 (n=5)	50.5±3.6 (n=6)	36.9±1.5 (n=5)	13.6±1.3 (n=5)	13.1±1.5 (n=6)	1.7±0.0 (n=6)
<i>t</i> Test						
1a-1b	P<0.001	P<0.05	P<0.02	P<0.01	n.s.	n.s.
2a-b	n.s.	n.s.	n.s.	P<0.001	n.s.	n.s.
1a-2a	P<0.01	P<0.01	n.s.	P<0.001	P<0.02	n.s.
1b-2b	n.s.	P<0.002	P<0.01	P<0.01	n.s.	n.s.

Values are mean ± 1 S.E. with number of rats per group in parentheses.

sample method<sup>10</sup> (Aperia & Herm 1975). Nucleotide counting was performed in a Packard Auto-Gamma Spectrometer.

**Arterio-venous pressure hematocrit.** The arterial blood pressure ( $P_A$ ) was determined by connecting the arterial catheter to a Hewlett Packard pressure recorder. Renal venous pressure ( $P_V$ ) was measured following puncture of the renal vein with a sharp needle connected to the pressure recorder. The arterial hematocrit (Hct) was determined by centrifuging blood in heparinized glass capillaries at 10 000 rpm for 5 min.

#### Abbreviations

C Arterial plasma protein concentration  
P<sub>oc</sub> Hydrostatic pressure in glomerular capillaries

P<sub>r</sub> Hydrostatic pressure in tubules during free flow  
P<sub>AA</sub> Ultrafiltration pressure in afferent parts of glomerular capillaries  
SFP Stop flow pressure  
 $\Pi_{AA}$  Oncotic pressure in afferent arterioles  
RVR Renal vascular resistance

#### Equations

- 1  $P_{AA} = SFP - P$
- 2  $P_{oc} = SFP + \Pi_{AA}$
- 3  $RVR = (P - P_V) / RBF$
- 4  $\Pi_{AA} = 2.1C + 0.16C + 0.009C$

The Student's *t*-test was used for data analysis. Probability values of less than 0.05 were regarded as significant.

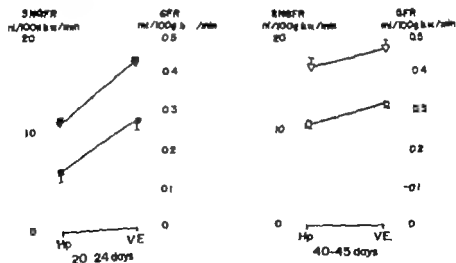


Fig. 4 Changes in SNRFR (squares) and GFR (triangles) induced by volume expansion in 20 to 24-day-old rats (filled symbol) and 40 to 45-day-old rats (unfilled symbol).

Age	P (mmHg)	P <sub>AA</sub> (mmHg)
2-13	3.5±0.4 (n=5)	16.8±1.0 (n=11)
2-19	2.6±0.7 (n=5)	13.7±5.6 (n=5)
4-16	4.5±0.8 (n=3)	21.3±1.7 (n=6)
10-14	3.2±0.7 (n=3)	23.9±3.1 (n=6)
	P<0.001	s. s.
	P<0.001	P<0.001

## RESULTS

The degree of saline expansion was assessed by determination of the hematocrit (Hct) and the serum protein concentration ( $C_A$ ) (Fig. 1). Although the hematocrit and the serum protein concentration were consistently lower in younger than in older rats, the infusion of isotonic saline to an amount corresponding to 6% of b wt. resulted in an equivalent decrease in both parameters in both age groups.

Renal blood flow (RBF) and glomerular filtration rate (GFR) are shown in Table 3. During hydropenia, both RBF and GFR were higher in older than in younger rats. The differences were significant both in absolute terms and when values were related to body weight.

During volume expansion RBF and GFR increased in both age groups. With an equivalent degree of volume expansion, however, the increase in RBF and GFR were more pronounced in younger than in older rats. When the GFR and RBF are related to body weight there was no significant difference between 20- and 24-day-old rats and 40- and 45-day-old  $VE_{4\%}$  rats. The relation between changes in hematocrit and RBF and GFR are shown in Fig. 2. Since all groups of rats are included in this plot the hematocrit values for young and old rats overlap. It is apparent that the fall in hematocrit induced by volume expansion is associated with

sharper rise in renal blood flow and GFR in younger than in older rats.

Renal vascular resistance (RVR) was determined (Eq. 3) in hydropenic and  $VE_{4\%}$  rats of both age groups (Fig. 3). During hydropenia, RVR was almost 1.5-fold higher in younger than in older rats.  $VE_{4\%}$  resulted in a more pronounced fall in RVR in younger (1.7-fold) than in older rats (1.1-fold).

The results of the micropuncture studies are shown in Table 2. In hydropenic rats, the SNGFR in absolute terms as well as in relation to b wt. was significantly lower in younger than in older rats. During volume expansion, SNGFR increased more in younger than in older rats. It is evident that the age dependent as well as the hydration dependent changes in SNGFR and total GFR paralleled each other (Fig. 4). Table 2 includes the results from the intrarenal pressure recordings in young and adult hydropenic rats which are in complete agreement with previous findings (Allison et al. 1977). During hydropenia stop flow pressure (SFP) and hydrostatic pressure in the glomerular capillaries ( $P_{GC}$ ) as well as the ultrafiltration pressure at the beginning of the glomerular capillaries ( $p_{iAA}$ ) were lower in younger than in older rats. In the  $VE_{4\%}$  animals, these differences between young and old rats became more pronounced, since  $VE_{4\%}$  induced a significant fall both in  $P_{GC}$  and SFP in young rats but had no effect on these parameters in older rats. The pressure in the efferent arterioles ( $P_{eA}$ ) was the same in all groups. The arterial blood pressure was lower in younger than in older hydropenic rats.  $VE_{4\%}$  had no characteristic effect on arterial blood pressure in either age group.

## DISCUSSION

The results of the present study show that an equivalent degree of volume expansion (VE) results in a more pronounced increase in RBF and GFR in the immature than in the mature kidney. An enhanced effect of VE on GFR in the human neonate has also been observed but it has usually not been discussed (Broberger 1973; Brodeur et al. 1972; Leake et al. 1976). The finding that the immature kidney is more sensitive to vasodilatation caused by VE suggests that the immature renal vascular bed is markedly constricted during basal conditions. The results of this study therefore provide further evidence for the hypothesis that the low RBF during early postnatal

life is due to physiological rather than to anatomical factors (José et al 1974 Tucker & Blantz 1977)

Although a positive salt and fluid balance results in a relatively larger increase in GFR in younger than in older rats the implications of these findings for the acute control of fluid and electrolyte homeostasis remain uncertain

A VE corresponding to 6% of b wt induced not only a pronounced but also a parallel increase in RBF and GFR in the young animal. This provides further insight into some of the factors that govern the GFR and which are of particular importance during late postnatal development. Since vasodilatation caused by VE may acutely increase the GFR almost 2 fold and result in GFR values that do not differ from those in the adult animal when related to b wt it is unlikely that the low water permeability of the glomerular capillaries is of importance in lowering the GFR at this developmental stage.

The second part of this study was devoted to a more detailed examination of the dynamics of nephron filtration during hydropenia and VE in the developing rat. In the superficial nephron accessible to micropuncture the SNGFR increased to a similar extent as the total GFR (Fig. 4). This indicates that no redistribution of GFR takes place during the transition from hydropenia to VE. The stop flow pressure, glomerular hydrostatic pressure and ultrafiltration pressure in the afferent end of the glomerular capillaries were somewhat lower in hydropenic young rats than in hydropenic old rats. The difference in  $P_{GC}$  between 20 to 24 and 40 to 45-day-old hydropenic rats was however much less pronounced than the previously observed difference in  $P_{GC}$  in the neonatal and adult guinea pig (Spitzer & Edelmann 1971). Thus although an increase in the hydrostatic component of the ultrafiltration pressure may well explain the early postnatal rise in GFR in guinea pigs it is probably of minor importance for the late postnatal rise in GFR in rats.

It is noteworthy however that while a 6% volume expansion resulted in an almost 2 fold increase in SNGFR it actually decreased the hydrostatic pressure in the glomerular capillaries in young rats. Thus by exclusion it can be concluded that glomerular blood flow must be a major determinant of the GFR during acute changes in fluid balance in the 20 to 24-day-old rat. It seems likely that renal blood flow exerts its effect on the GFR not only by presenting more plasma fluid/unit time to

the filtering capillaries but also by changing the oncotic pressure along the length of the glomerular capillaries. It is of interest in this connection that in a previous study from this laboratory it was shown that during postnatal development from 20 to 40 days of age the GFR also increases mainly as a function of RBF (Aperia & Herm 1973).

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Portions of these studies were presented at the XVIIIth International Congress of Physiological Sciences in Paris July 1977, the Fourth International Symposium of Pediatric Nephrology at Helsinki Aug 1977 and at the Third European Colloquium on Renal Physiology June 1979 and have been published in abstract form.

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## Cholinergic vasoconstrictor effects in the rabbit eye Vasomotor effects of pentobarbital anesthesia

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BILL, A. & STJERNSCHANTZ, J. Cholinergic vasoconstrictor effects in the rabbit eye. Vasomotor effects of pentobarbital anesthesia. *Acta Physiol Scand* 1980, 108, 419-424. Received 1 Aug. 1979. ISSN 0001-6772. Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden.

Oculomotor nerve stimulation causes vasoconstriction in the anterior uvea, which is due partly to muscarinic mechanism and partly to non-sympathetic aminergic mechanism. The labelled microsphere method was used to analyze the effect of pentobarbital anesthesia on the resting cholinergic vasomotor tone in the anterior uvea and to determine the relationship between stimulation frequency and vasomotor response. An attempt was made also to ascertain whether the aminergic part of the vasoconstriction is caused by release of 5-hydroxytryptamine or norepinephrine. Induction of pentobarbital anesthesia caused marked vasodilation in the iris and the ciliary processes and subsequent muscarinic blockade had no effect on the blood flow. A similar result was obtained in the optic nerve. In the choroid plexus, heart muscle, pineal body and cecum, pentobarbital anesthesia caused vasodilation and a subsequent muscarinic blockade caused vasoconstriction. In the brain pentobarbital anesthesia caused marked reduction in the blood flow of the grey matter and moderate reduction in the white matter. After a muscarinic blockade there was some increase in the blood flow of the grey matter. Stimulation of the oculomotor nerve caused near maximum vasomotor responses at 10-20 Hz; maximum effect on the pupil size was obtained at 40-50 Hz. Depletion of 5-hydroxytryptamine with fenfluramine did not prevent the aminergic part of the vasoconstriction and marked vasoconstriction was also observed after pretreatment with reserpine. The results indicate that pentobarbital anesthesia abolishes most of the spontaneous cholinergic vasoconstrictor tone of the anterior uvea and that the aminergic part of the oculomotor nerve induced vasoconstriction is caused by the stimulation of phenolamine-sensitive receptors by a mechanism probably not involving release of norepinephrine or 5-hydroxytryptamine.

**Key words:** Pentobarbital anesthesia, oculomotor nerve stimulation, cholinergic vasoconstriction, blood flow.

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## Cholinergic vasoconstrictor effects in the rabbit eye Vasomotor effects of pentobarbital anesthesia

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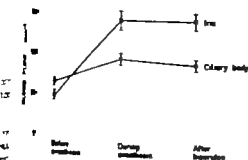


Fig 1

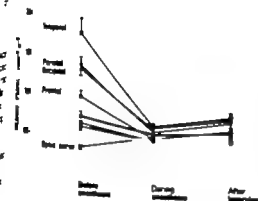


Fig 3

$123 \pm 1.6$  kPa,  $4.6 \pm 0.4$  and  $7.31 \pm 0.05$  respectively. Biperiden had no significant effect on the blood pressure or the acid-base balance.

Fig 1 shows results for the anterior uvea. Barbiturate anaesthesia markedly increased the blood flow in the iris and ciliary body ( $P < 0.001$ ). Biperiden then had practically no effect. Fig. 2 shows vasodilating responses to pentobarbital anaesthesia in some other tissues. Pentobarbital sodium increased the blood flow in the pineal body, choroid plexus and large intestine ( $P < 0.005$ ) and tended to do so also in the heart muscle ( $P < 0.05$ ). After biperiden the flow decreased in all these tissues ( $P < 0.01$ ). Fig 3 shows that in the brain there was a marked reduction in the blood flow of the grey matter after the induction of anaesthesia. The reduction was highly significant ( $P < 0.001$ ) in all parts. After biperiden there was a small but statistically significant ( $P < 0.01$ ) increase in the blood flow in the white matter. Pentobarbital reduced the blood flow moderately ( $P < 0.01$ ) and biperiden had no significant effect. In the optic nerve pentobarbital anaesthesia increased the blood flow ( $P < 0.01$ ) but biperiden had no statistically significant effect.

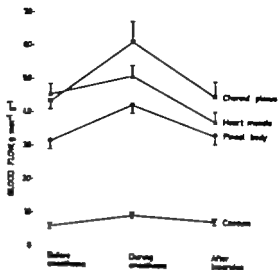


Fig 2

Figs 1-3 Effect of pentobarbital anaesthesia and subsequent administration of biperiden on regional blood flow. Values shown are mean  $\pm$  S.E., number of animals = 9. In Fig 3 each region of the brain is represented by one symbol, filled for grey matter, unfilled for the white.

#### Oculomotor nerve stimulation

The effect of oculomotor nerve stimulation on the blood flow in the anterior uvea is presented in Table 1. The reduction in blood flow in the iris was greater than that in the ciliary processes. The results were similar in the pigmented and albino rabbits.

Fig 4 shows the relationship between the stimulation frequency and the response in blood flow.

Table 1 Blood flow in the anterior uvea ( $M \pm S.E.$ ) on the stimulated and control side. The flow on the stimulated side was expressed also as percent of control flow.

Means ( $\pm$  S.E.) of the percentage values are shown. \* indicates statistically significant reduction at the  $P < 0.01$  level and \*\*\* at the  $P < 0.001$  level ( $n = 10$ ).

Tissue	Stimulated side (mg/min)	Control side (mg/min)	Stimulated side % of control
Iris	$39 \pm 8$	$138 \pm 11$	$33 \pm 7^{***}$
Ciliary processes	$57 \pm 9$	$82 \pm 10$	$72 \pm 7^{**}$
Anterior uvea	$95 \pm 12$	$220 \pm 33$	$50 \pm 7^{***}$



dures involved in stereotaxic stimulation had abolished a vasoconstrictor tone in the anterior uvea. A cholinergic blockade under these conditions had only a moderate vasodilating effect in the anterior uvea. Previous studies on the effects of barbiturate anesthesia on regional blood flow in rabbits did not include determinations of the blood flow in the eye (Aardal, Svanes & Egenberg 1973).

The purpose of the present study was to investigate further the mechanisms involved in the oculomotor nerve control of the uveal blood flow in rabbits. The aims were: 1) to determine if pentobarbital anesthesia affected vasomotor tone in the uvea and some other tissues; 2) to determine the stimulation frequency response relationship of cholinergic vasoconstriction; and 3) to try and analyze if depletion of the monoamines affected the response to oculomotor nerve stimulation appreciably.

## MATERIALS AND METHODS

### 1. Effect of pentobarbital anesthesia and cholinergic blockade

The experiments were performed in albino rabbits of both sexes and weighing 1.8–3.2 kg. One day before the actual experiment one femoral artery was cannulated with a polyethylene tubing and the left heart ventricle was cannulated via the left subclavian artery using another tubing. Cannulation was performed under pentobarbital anesthesia and the animal recovered within a few hours. On the next day the animal was placed in an open box and about 1–2 million radioactively labelled  $^{15}\mu\text{m}$  microspheres (3M Company, St. Paul) in 1 ml saline were injected into the left ventricle over 10 s. From the start of the injection and for the next 60 s the blood from the cannulated femoral artery was collected. The flow rate was about 1 ml/min. Anesthesia was then induced with pentobarbital sodium, about 50–60 mg/kg b.wt. The anesthetic was given slowly i.v. and at a dose which made it possible to put the animal on its back and pinch the ear without marked reactions. After 15 min of anesthesia a new blood flow determination was made using microspheres labelled with another isotope. Immediately after the second flow determination cholinergic blockade was accomplished by i.v. injection of biperiden. A third flow determination was performed 5 min later using microspheres labelled with a third isotope. Details of the technique used for flow measurements were described previously (Sjörschantz et al. 1976).

### 2. Oculomotor nerve stimulation

Albino as well as pigmented rabbits of either sex and weighing 1.7–4 kg were used. The animals were anesthetized with pentobarbital sodium. Initial dose 40–60 mg/kg b.wt. Additional doses were injected when necessary to maintain anesthesia. All the animals received tubocurarine 0.5–1 mg/kg b.wt. before stimulation. The

animals were artificially ventilated and  $P_{O_2}$ ,  $P_{O_2}$  and pH of the arterial blood were checked. Both femoral arteries were cannulated: one for blood sampling and the other for blood pressure measurement. The animals were thoracotomized and the left heart ventricle was cannulated. A mixture of Macrodex (Pharmacia, Uppsala) and isotonic saline (1:1) was injected intravenously to increase the blood pressure whenever needed. The amount of this mixture did not exceed 40 ml. The animals were kept warm by a heating pad.

**Intracranial stimulation of the oculomotor nerve.** The intracranial part of the oculomotor nerve was electrically stimulated with a stereotaxic technique (see Sjörschantz 1976; Sjörschantz et al. 1976). The nerve was stimulated about 3–4 mm posterior to the place where it leaves the cranial cavity. Electrical stimulation was carried out using a bipolar electrode with unidirectional square wave pulses. Intensity 3–6 V, duration 1 ms and frequency ranging from 5–90 Hz, mostly being 40–50 Hz. In each experiment a first stimulation to check the position of the electrode was performed with a 40–50 Hz frequency 10–15 min before the first flow determination. Only experiments with good myosis immediately reversible after stimulation were accepted. The nerve was stimulated for 2 min before the blood flow determination and during it. After the experiment the exact position of the electrode was checked.

**Pharmacologic treatment.** Cholinergic blockade was induced by biperiden lactate 3–5 mg/kg b.wt. The lower dose completely inhibited the response of the sphincter pupillae muscle to oculomotor nerve stimulation. Fenfluramine chloride was daily administered i.m. 20–40 h before stimulation in a dose of 10 mg/kg b.wt. (each day) in order to deplete 5-hydroxytryptamine stores in a selective way. At this dosage fenfluramine causes marked depletion of brain serotonin (Dubaut & Verdavanne 1967). An additional dose of 5 mg/kg b.wt. was given i.m. 3–5 h before stimulation. Reserpine was administered i.m. 10–14 h before stimulation in a dose of 5 mg/kg b.wt. At this dosage reserpine causes marked depletion of monoamines (Shore, Olin & Brodie 1957) which resulted in a low blood pressure. An additional dose of 5 mg/kg b.wt. was given i.m. 3–5 h before stimulation.

## RESULTS

### Effect of pentobarbital anesthesia and cholinergic blockade

Preliminary experiments indicated that induction of pentobarbital anesthesia had little effect on the blood flow in most tissues, caused vasoconstriction in some and dilation in others. Some of the tissues in which there seemed to be a change were subjected to a more detailed investigation in a series of nine animals. Before anesthesia the mean arterial blood pressure  $P_{O_2}$ ,  $P_{O_2}$  and pH in this series were  $84 \pm 3$  mmHg,  $13 \pm 1.5$  kPa,  $4.3 \pm 0.4$  kPa and  $7.38 \pm 0.02$  respectively. After 10–15 min of anesthesia the corresponding values were  $74 \pm 3$  mmHg

was in the blood vessels of the anterior uvea. A corresponding, almost complete elimination of the activity in the nerve fibres supplying the sphincter muscle cannot be expected. The pupil is wide after peribulbar anesthesia but it reacts to light and infrared light—as used in the present experiments—can thus be expected to cause some activity in the sphincter fibres.

#### *Effects of oculomotor nerve stimulation*

The results of the experiments with oculomotor nerve stimulation at different frequencies indicates that the vasomotor effect has a frequency-response relationship very different from that in the sphincter muscle: near maximum vascular effects are produced already at frequencies between 10–20 Hz. To produce near maximal responses in the sphincter to pupile frequencies around 40–50 Hz had to be added to the spontaneous activity which probably is low.

As mentioned in the introduction vasoconstrictive effects of acetylcholine have been obtained in *in vitro* preparations of blood vessels. And such effects have been observed also in artificially perfused organs. If *in vitro* preparations of arteries are given a local  $\alpha_1$  by norepinephrine acetylcholine leads to  $\alpha_1$  constriction which suggests that there are receptors in the smooth muscles for dilation as well as for constriction. Under *in vivo* conditions there is still another effect of acetylcholine: it tends to reduce the amount of norepinephrine released by the sympathetic nerves—an effect that contributes to making the *in vivo* response dilatory (Vimouste 1977). Usually the *in vitro* vasoconstrictive effects of acetylcholine as well as the dilative effects are muscarinic but vasoconstrictor effects that could not be blocked by atropine have been reported for the perfused rabbit ovarian artery (Graham & Sano 1971).

In the perfused cat eye acetylcholine as well as stimulation of the ciliary ganglion has been reported to produce effects indicating vasoconstriction in the vein. These effects could be blocked by cholinergic blocking agents as well as by adrenergic  $\alpha$ -receptor blocking agents and were interpreted to indicate release of norepinephrine from intraocular neuronal terminals (Macri & Cevano 1975).

Previous studies (Sjernerchantz & Bill 1979) indicated that in cats and monkeys oculomotor nerve stimulation under *in vivo* conditions causes vasodilation in the ciliary body and vasoconstriction in the

iris. The responses in rabbits were constrictive as in the present experiments. The iris and the ciliary processes have a rich supply of sympathetic nerves, and stimulation of these nerves causes marked vasoconstriction (Sjernerchantz et al 1977). Prerequisites thus seem to exist for one of the mechanisms by which acetylcholine could cause vasodilation. And the response in the ciliary body in cats and monkeys may be secondary to inhibition of the norepinephrine release or/and stimulation of vasodilating receptors. But in the iris—and in rabbits also in the ciliary body—the normal response seems to be dominated by stimulation of the constrictive receptors.

Previous studies (Bill et al 1976; Sjernerchantz et al 1977) indicated that the vasoconstriction in the anterior uvea in rabbits was due partly to a cholinergic and partly to an adrenergic mechanism. Vasoconstriction induced by oculomotor nerve stimulation in sympathectomized rabbits ruled out the possibility of a sympathetic co-stimulation (Alm, Sjernerchantz & Bill 1976). This has been confirmed further by experiments in which sympathetic blockade has been carried out with bretylium prior to stimulation (unpublished data). It never became clear in the previous studies whether the adrenergic mechanism involved receptors only at the target area, or whether there was also a local release of a biogenic amine as suggested originally by Macri & Cevano (1975). From our previous experiments (Sjernerchantz et al 1977) 5-hydroxytryptamine seemed a possible candidate. Since in the present experiments fenfluramine which is a selective 5-hydroxytryptamine depletor in the brain and in platelets (Picotti et al 1977) was totally without effects in preventing the adrenergic part of the vasoconstriction, this hypothesis received no support. It is not clear at present if there are any 5-hydroxytryptamine nerves in the uvea and whether they would be depleted to an appreciable extent by fenfluramine. Therefore also reserpine was used in depletion experiments, the aim being to deplete adrenergic nerves containing norepinephrine, 5-hydroxytryptamine or dopamine. The vasoconstrictive effect of stimulation in reserpinized animals differs markedly from the effect in phentolamine treated animals in which stimulation caused vasodilation in the ciliary body (Bill et al 1976). And the effect of stimulation after biperiden in reserpinized animals was similar to that obtained previously with biperiden only. Thus neither reserpine nor fenfluramine

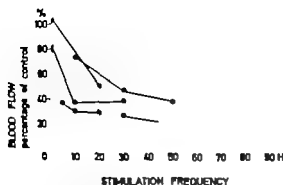


Fig. 4 The relationship between stimulation frequency and the iridal blood flow

Marked effects on the blood flow were observed already at 5–10 Hz. At these frequencies there was little change in the pupil diameter. Maximum miosis was obtained only at 40–50 Hz and higher.

Rabbits pretreated with fenfluramine showed a distinct vasoconstrictive response to stimulation in spite of a cholinergic blockade (Fig. 5). The mean arterial blood pressure after fenfluramine and biperiden before stimulation was  $81 \pm 4$  mmHg and during stimulation  $79 \pm 7$  mmHg. Pretreatment of the animals with reserpine was in a similar way without effect in blocking the vasoconstrictive response to stimulation (Table 2). Most of the vasoconstriction could be abolished by an additional cholinergic blockade (Table 2) but there remained a probably significant effect in the iris. The mean arterial blood pressure after reserpine was  $62 \pm 3$  mmHg and after reserpine and biperiden  $62 \pm 3$  mmHg.

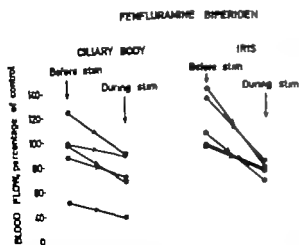


Fig. 5 Effect of oculomotor nerve stimulation on the blood flow of the iris and the ciliary processes in rabbits pretreated with fenfluramine and biperiden.

Table 2 Effect of oculomotor nerve stimulation on the blood flow in the anterior uvea in reserpine-treated animals

The blood flow is expressed as percentage of the blood flow in the control eye. \* indicates a statistical significance at the  $P < 0.05$  level, \*\* the  $P < 0.01$  and \*\*\* the  $P < 0.001$  level ( $n = 10$ ) ( $M \pm S.E.$ )

	Iris (%)	Ciliary proc. (%)	Anterior uvea (%)
Pretreatment with reserpine	$40 \pm 7$	$76 \pm 7$	$57 \pm 6^{**}$
Pretreatment with reserpine and cholinergic blockade with biperiden	$79 \pm 8$	$94 \pm 8$	$85 \pm 7$

## DISCUSSION

### Effects of pentobarbital and biperiden

Some of the effects of the pentobarbital anaesthesia shown in Fig. 1–3 have been observed previously. Goldman & Sapirstein (1973) reported that brain blood flow was reduced in rats as a consequence of pentobarbital anaesthesia and that there was a correlation between preanesthetic values and the reduction in flow. As in the present experiments, anaesthesia tended to reduce blood flow to a relatively uniform level. The changes observed in the present study in the choroid plexus and pineal body are puzzling. The pineal body has been suspected of being involved in stabilizing and deactivating processes in the brain (Romijn 1978). Increased flow may be the result of increased activity in this particular part of the brain. The observation that after cholinergic blockade pineal and choroidal plexus blood flow decreased and that at the same time there was an increased flow in the grey matter raises the question whether there may be a functional connection between the pineal body and the rest of the brain involving a cholinergic mechanism. The reason for increased blood flow in the large intestine after pentobarbital is not clear. The reduction in flow caused by the cholinergic blockade suggests that in this tissue there was a cholinergic vasodilating tone after the induction of anaesthesia.

The observation that in the anterior uvea pentobarbital anaesthesia caused a very marked increase in blood flow and that a subsequent cholinergic blockade had no effect indicates that the anaesthesia virtually abolished the spontaneous cholinergic

# Effects of sprint and endurance-training on capillary circulation in human skeletal muscle

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Serious approaches have been used to clarify the effect of endurance-training on structural, biochemical and physiological properties of skeletal muscle. A number of investigators (Grimby et al 1967, Camen et al 1969, Vasmataz et al 1970) have shown that blood flow per kg of working muscle, as measured by  $^{133}\text{Xe}$  clearance method at submaximal work level, is slower in trained individuals than in controls. Trained individuals show greater intramuscular oxygen difference during submaximal exercise, which is presumably due to increased capacity of the trained individuals to extract oxygen from circulation (Saltin et al 1969). We recently showed increased capillary permeability in endurance-trained athletes which is most likely a manifestation of increased capillary surface area (Leinonen et al 1978). This finding is consistent with morphological studies which showed increased muscle capillary density in endurance-trained athletes (Andersen 1975, Brodal et al 1977, Ingjer 1979). In contrast to endurance-training little is known about the consequences of sprint-training. Therefore muscle blood flow (MBF) and capillary diffusion capacity (CDC) were estimated by measuring local distances of  $^{133}\text{Xe}$  and  $^{125}\text{I}$  after ischemic exercise in a group of sprinters, and the results are compared with the data for distance runners and controls.

**Subjects and method** 6 international-caliber sprinters (3 were in the Finnish Olympic track team) were studied. They had been actively training and competing for at least 3 years. Their main training program took the form of short duration daily exercises such as sprinting, interval running and weight lifting. In addition, they had a relatively light endurance program of 15-35 km of running per week. The 3 distance runners (3 were Olympic medalists and the double European champion) ran regularly 70-135 km per week. All athletes were studied during their most active competition season. The 10 controls were sedentary adult volunteers. The methods for measurement and calculation of MBF

and CDC were in detail described elsewhere (Leinonen et al 1978). Briefly the clearance rates were recorded with the use of lipophilic  $^{133}\text{Xe}$  (50-70  $\mu\text{Ci}$ ) and hydrophilic  $^{125}\text{I}$  (10-70  $\mu\text{Ci}$ ) dissolved in 0.1 ml of isotonic saline deposited locally with the use of a narrow gauge needle in the anterior tibial muscle made hyperemic with ischemic exercise. The rate constants and CDC for  $^{125}\text{I}$  were calculated for the initial rapid wash-out slope (0-30 s after onset of post-exercise hyperemia) and the secondary "steady-state" wash-out slope (30-90 s) of the clearance curve. MBF was calculated from the period of maximal hyperemia (30-90 s after onset of post-exercise hyperemia).

**Results** The results, as well as statistical analyses, are given in Table 1. The sprinters had only a slight, nonsignificant increase in MBF (10%) and in CDC (13%) when compared to controls. MBF and CDC were significantly greater in distance runners than in sprinters or controls. The average increment of MBF was 34% and that of CDC nearly 50%.

**Discussion** Being a lipophilic gas  $^{133}\text{Xe}$  freely crosses cell membranes the main hindrance to its exchange with blood being the diffusion distances while the exchange of lipid-insoluble substances such as  $^{125}\text{I}$  is restrained by capillary membranes. The increases of MBF and CDC (particularly during the secondary slope of  $^{125}\text{I}$  clearance curve) in distance runners are explained by an increase in the muscle capillary surface area, as discussed earlier (Leinonen et al 1978). This is also supported by recent studies which showed a marked increase in muscle capillary supply when untrained and endurance-trained groups were compared (Andersen 1975, Brodal et al 1977, Ingjer 1979) or when the same subjects were studied before and after training (Andersen & Henriksson 1977). Furthermore Ingjer (1978) showed that the increase in capillary supply occurs linearly with increasing maximal aerobic power. The observed nearly 50% increase

gave results mimicking those of receptor blockade with phentolamine. It seems unlikely then that oculomotor nerve stimulation normally results in release of any of the amines mentioned or enhances the effect of some circulating amine. Other explanations such as effects of acetylcholine on the aminergic receptors or release of an unconventional transmitter with effects on the vascular aminergic receptors or peculiarities in the acetylcholine receptor structure in the smooth muscle would seem to be more likely alternatives.

We would like to thank Ms Siv Nilsson and Ms Monica Lindquist and Ms Monica Thoren for technical assistance. This study was supported by grant B78-14X-00147 14B from the Swedish Medical Research Council and grant SROIE00475 11 from the National Eye Institute U.S. Public Health Service. The study was also supported by a grant from the Finnish Academy of Sciences to JS.

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Table 1 Average data for controls, sprinters and distance runners (mean  $\pm$  S.D.)

Figures within parentheses denote number of subjects. ns=non significant

Groups	Age (yrs)	Muscle blood flow (ml 100 g min <sup>-1</sup> )	Rate constant for <sup>133</sup> I (min <sup>-1</sup> )		Capillary diffusion capacity for <sup>133</sup> I (ml 100 g min <sup>-1</sup> )	
			Initial slope	Secondary slope	Initial slope	Secondary slope
Controls (10)	27.8	62.9 $\pm$ 8.7	0.6 $\pm$ 0.17	0.35 $\pm$ 0.07	11.3 $\pm$ 3.5	5.6 $\pm$ 1.3
Sprinters (6)	23.3	68.9 $\pm$ 14.4	0.81 $\pm$ 0.18	0.39 $\pm$ 0.03	14.5 $\pm$ 3.4	6.3 $\pm$ 0.6
Distance runners (13)	26.4	84.1 $\pm$ 15.1	0.88 $\pm$ 0.22	0.51 $\pm$ 0.07	15.9 $\pm$ 4.4	8.3 $\pm$ 1.1
<i>Statistical analysis*</i>						
Controls vs. sprinters	ns	ns	ns	ns	ns	ns
Controls vs. distance runners	P<0.001	P<0.005	P<0.001	P<0.001	P<0.001	P<0.001
Sprinters vs. distance runners	ns	ns	P<0.001	ns	P<0.001	P<0.001

Student's *t*-test

of capillary permeability in endurance trained athletes corresponds quite well with the results for maximal oxygen uptake and capillary supply. Brodal et al. (1977) found that the mean capillary density of skeletal muscle and maximal oxygen uptake were both 40% higher in endurance-trained individuals than in untrained and in Ingjer's (1979) recent study the percentages were 49% and 56% respectively. These adaptations to endurance training together with increased oxidative enzyme activity (Varnauskas et al. 1970; Andersen & Henriksson 1977) and capillary permeability, i.e. increased transcapillary transport of small molecules, all compensate for the lower blood flow measured during dynamic exercise, thus securing adequate muscle nutrition.

There is however only scarce data dealing with the adaptive response of human muscle to anaerobic sprint training. Sprinters and distance runners have distinct muscle fiber compositions, the latter having a higher percentage of type I (slow twitch) fibers (Costill et al. 1976). This is mostly due to genetic factors and muscle fiber composition probably predetermines athlete's success in either sprint or distance running. Jansson and coworkers (1978) however showed that the relative proportion of type I fibers in trained individuals is also decreased by a period of interval training. Type I fibers are supplied with the greatest number of capillaries around each fiber, also relative to fiber area (Ingjer 1979). This indirect evidence points to the fact that sprint training does not affect muscle capillary supply to a same degree as endurance

training. This is also reflected in the present observation that MBF and CDC were only slightly increased in sprinters.

In conclusion, additional studies are needed to describe the changes occurring in muscle due to training, especially various stages of sprint and endurance training. In addition to structural and histochemical studies, the functional changes can easily and noninvasively be studied using the krypton isotope clearance method.

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## Acetylcholinesterase positive nerves in the guinea-pig endometrium

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Nerves in the endometrium and nerve terminals to its glands and epithelial linings have been demonstrated by several authors in different mammals such as man, macaque rabbit, guinea-pig and rat with metal impregnation techniques and supra- or intra-vital acetylcholine blue staining, and also by electron microscopy (vide Hammarström & Sjöstrand 1979). Endometrial nerves have also been shown to be acetylcholinesterase (AChE) positive in man (Cajland 1962, 1969) and in rat (Adham & Schenk 1964). In the guinea-pig endometrium, however, no AChE positive nerves have been observed (Jordan 1971, Thorbert et al 1977).

Nonetheless, in a recent study we have obtained physiological and pharmacological evidence of a cholinergic secretory innervation of the guinea-pig endometrium (Hammarström & Sjöstrand 1979). Since the lack of demonstration of endometrial AChE positive nerves in the guinea-pig could be due to technical difficulties with regard to the composition of the endometrial tissue (vide Thorbert 1977), we have reinvestigated the matter.

**Material and methods** The modified Koelle fluorescent method of Karnovsky & Roots (1964) was used for identifying AChE positive nerves. Five guinea-pigs treated with estradiol benzoate (1 mg/kg) and hydroxyprogesterone caproate (150 mg/kg (Primoblaston®) every third day for 6-8 days were stunned and bled. The uterus was carefully excised out and cut into 5-8 mm pieces. The pieces were either directly frozen (-30°C) or frozen after 1-16 h of fixation in ice-cold formalin (Karnovsky & Roots) followed by 1-4 days in ice-cold paraffin solution (Holl, Hobbager & Pawan 1961). Some unfixed sections were postfixated in osmium according to El-Badawi & Schenk (1967). Sections (10 µm) were cut in a cryostat. Tissue in the incubation medium recommended by Karnovsky & Roots was 1-5 h. Iso-OMPA (4 µM) was used to inhibit nonspecific cholinesterase activity. For general staining of the sections eosin or lightgreen was used.

**Result comments and conclusions** As expected there were difficulties in demonstrating AChE positive nerves in the endometrium. The most satisfactory results were obtained in sections from pieces fixed for 1 h and incubated in the substrate medium for 3 h. No certain AChE activity was noted in sections from pieces fixed for more than 3 h. Unfixed or postfixated sections exhibited AChE activity but it was difficult to localize properly.

In accordance with earlier studies (Bell 1968, Thorbert et al 1977) AChE-positive nerves were observed in the parametrial arteries (Fig. 1a). However, we could also trace such nerves into the myometrial branches (Fig. 1b). Also in the myometrium AChE positive fibres were seen (Fig. 1c). Furthermore AChE positive nerve trunks were observed in the endometrium, not only in the basal part but also in the functionals (Fig. 1d). Moreover they seemed to run free in the stroma and did not accompany arteries. However, it was not possible to discern distinct nerve terminals in the lining or glandular epithelium but terminals were noted close beneath (Fig. 1d and e).

The demonstration of AChE positive nerves in the endometrium supports our findings concerning uterine secretion, i.e. the endometrium has a cholinergic secretomotor innervation (Hammarström & Sjöstrand 1979). Nerves to the guinea-pig endometrial secretory cells have been described by Köstlin (1894) using silver impregnation of osmium tetroxide-potassium bichromate fixed material and by Jacobson & Nieves (1961) using intra-vital methylene blue staining. The terminals are however difficult to discern why the latter authors were forced to supply the methylene blue solution with hyaluronidase in order to decompose the stroma, for a clear staining and visualization of the terminals. Our study also emphasizes the well-known fact that in AChE histochemistry one must vary the conditions and techniques for each tissue and species in order to obtain suitable results.





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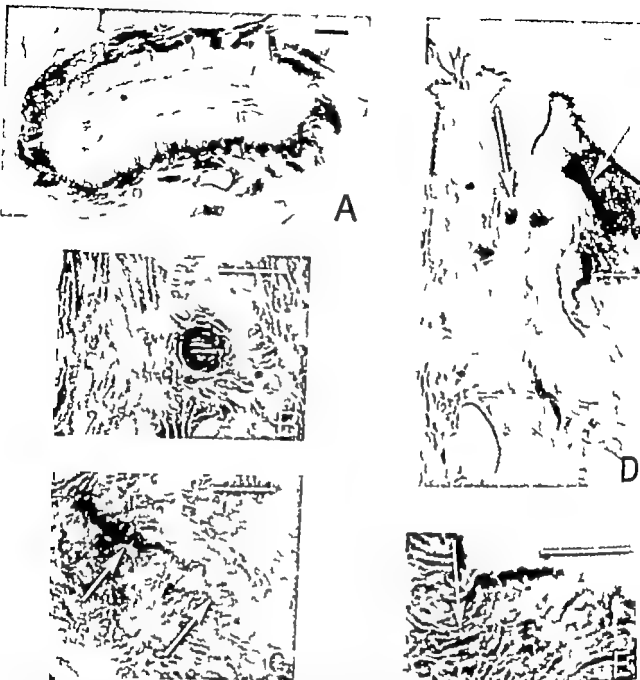


Fig 1 Acetylcholinesterase positive nerves in the guinea pig uterus. Technique of Karnovsky & Roots. General staining with eosin. Calibration line 10  $\mu$ m. (A) Intense AChE staining of nerve fibres in a parametrial artery. The fibres are mainly localized in the outer border of the media and in the adventitia. (B) Nerve fibres surrounding the smooth muscle in a small myometrial artery. (C) Nerve running in the myometrium (not adjacent to larger blood vessels). (D) Nerve trunks and fibres in the endometrium. Note the fibres close to glandular epithelium (thin arrows) and larger transversally sectioned trunks below surface epithelium (thick arrows). (E) Small terminal beneath epithelial lining of the endometrium.

Presumably the inability of Jordan (1970) and Thorbert et al (1977) to trace AChE positive nerves within the uterus was simply due to technical matters.

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insulin-sensitive receptors involved in water and salt balances

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Studies performed in the goat imply that mineral receptors regulating water intake and the release of vasopressin (AVP) have a paraventricular location and that the activity of these receptors is positively correlated to the osmolarity of the cerebrospinal fluid (CSF) (cf. Andersson 1978). McKinley, Denton and Wei (1978) suggested that there might be both osmosensitive and originally proposed by Verney (1947) of sodium sensitive receptors. The latter hypothesis was partly based on the observation that short infusions of sucrose or artificial CSF into the lateral ventricle elicited drinking and antidiuresis. It is to a much less extent than similar infusions of equiosmolar NaCl. In this study, we made prolonged intraventricular infusions of sucrose/NaCl and mannitol/NaCl. Intraventricular infusions were equiosmolar pure water and NaCl solutions. Both the osmotic and antidiuretic effects were absent during infusions of mannitol/NaCl. Sucrose/NaCl plasma AVP initially rose slightly and then fell in response to these infusions. It dropped during mannitol/H<sub>2</sub>O and rose markedly during hypertonic NaCl.

The osmotic composition of mannitol/NaCl sucrose/NaCl revealed a 10% lowering of the CSF Na<sup>+</sup> which could explain in why the diuretic and antidiuretic effects were much weaker and less persistent as compared to the effect of hypertonic NaCl. Changes in CSF Na<sup>+</sup> have been shown to be important in the regulation of renal sodium excretion (Andersson, Dallman & Olsson 1969) and recently also in the regulation of thirst (Wei, Ling, et al. 1979). Lowering of CSF Na<sup>+</sup> doubled the concentration of Na<sup>+</sup> in sodium deprived sheep where an increased CSF Na<sup>+</sup> diminished Na<sup>+</sup> uptake in the animal. It is concluded that sodium sensitive receptors not only participate in the regulation of water balance but also in the control of salt balance.

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## C2

### pH-dependence of receptor binding of insulin to rat adipocytes

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Binding of insulin to receptors in adipocytes is approximately 6 times higher at pH 7.5 compared to the binding at pH 6.5. A further increase in pH from 6.5 to 7.5 causes a light decrease in the binding of insulin. The aim of this study was to investigate the possible reasons for this change in binding. We have previously reported that about 70% of the receptor-bound insulin is released from the cell as degradation products (Gliemann & Sonne 1978). As the fractional degradation of receptor-bound insulin was the same in the investigated adipocytes (6.5 & 7.5) this could not explain the observed difference. It was also noticed that the difference in affinity due to the use of iodinated insulin (125I-125I-monomiodoinsulin (Gliemann et al. 1979)) gave identical results. This showed the same pH-dependence for both binding experiments. It is concluded that the observed binding difference was due to a change in affinity of the binding capacity. Kinetic experiments showed that the difference in binding

was unchanged and that the degree of negative cooperativity was small at all investigated pH values. Association experiments revealed that the decrease in affinity at alkaline pH could be accounted for by an increased association rate constant.

To test the biological consequences of the change in affinity we measured as well glucose incorporation into the lipid pool as 3-O-methyl-glucose transport (Whitesell & Gliemann 1978). The insulin dose-response curves for these biological parameters were accordingly shifted to the left at alkaline pH.

In conclusion, in the diabetic patient the acidosis will turn the cells insulin resistant and thereby make the sparse insulin even more unserviceable.

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only outward current and  $E_{Ca}$  in a 100 mV step

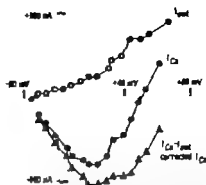
# RESULTS

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Large  $Ca^{2+}$  currents are important for activity in excitable cells. Measurements of the equilibrium potential for these currents give values between +40 and +65 mV. The  $Ca^{2+}$  equilibrium potential ( $E_{Ca}$ ) is at least 120 mV assuming that  $[Ca^{2+}]_i$  is  $5 \times 10^{-7}$  M. Several hypotheses exist to account for this discrepancy (Moolenaar & Spector 1979) for example that an only outward current interferes with the inward  $Ca^{2+}$  current.

For isolation of cell A of Helix aspersa with independent screened microelectrodes voltage depolarizations were made using conventional techniques (Stamnes 1975). During depolarizing step a cell displayed a fast inward current and delayed outward current. Ion substitution experiments showed that about 2/3 of the fast inward current is carried by  $Ca^{2+}$  ( $I_{Ca}$ ). The free Ringer  $Ca^{2+}$  relationship (Fig. 1) and the time to reach the  $Ca^{2+}$  current was obtained for 11 test potentials.  $I_{Ca}$  was then blocked by adding 15 mM  $Ca^{2+}$  to the Ringer (with 2 mM  $Ca^{2+}$ ) and a 1 V fast depolarization again. All inward current was now blocked and the current was small and after pulses equal to the previously used time to peak  $Ca^{2+}$  current for each test potential (Fig. 1). This curve shows the only outward current ( $I_{out}$ ) that contaminated  $I_{Ca}$ .

The equilibrium potential for early current was usually measured as the intercept of the 1 V



curves with the voltage axis ( $E_{Ca}$  intercept). In this case it is about +45 mV for  $I_{Ca}$ .  $E_{Ca}$  intercept (found by extrapolation) shifts considerably to the right when  $I_{Ca}$  is corrected by subtraction of  $I_{out}$ .  $I_{Ca}$  and  $I_{out}$  would be expected to unite at  $E_{Ca}$ . However they become nearly parallel at high potentials.  $I_{out}$  was insensitive to TEA (50 mM) or tetrodotoxin (0.5 mM). 4-aminopyridine (5 mM) caused little or no inhibition.

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## C6

Effects of and dependence on morphine  
in the spinal cord

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It has been proposed that development of morphine tolerance and dependence may be due to functional changes in monoaminergic neurons in the brain (May, Mo, Loh 1971). An alternative suggestion is that tolerance and dependence are related to changes in the sensitivity of opiate receptors (Cox 1978). Following transection of the spinal cord there is complete dual degeneration of monoaminergic neurons (Carlsson, Magnusson & Rosengran 1971). Transected rats were therefore used to investigate whether development of tolerance and dependence could be observed in structures deprived of monoaminergic input.

Complete spinal transections were made at the level of the tenth thoracic in 10 male rats. Sixteen rats were used in the study. After 14 days of training in a test situation 5 spinal rats and 8 control rats were injected with morphine 10 mg/kg i.p. At 12 hr interval 4 days later the rats were tested for sensitivity to morphine. The test situation with the tail flick showed all showed progressive reduction in drug effect (tolerance and dependence) during the period of morphine treatment.

Naloxone 500 (1 mg/kg) 100 min after the last morphine injection induced signs of withdrawal both in spinal rats (pronounced twitching and jerking of tail and hindlimbs) and in controls (jumping in increased defecation). Withdrawal was not precipitated by morphine and naloxone in transected and in intact rats not made tolerant to morphine.

It was concluded that morphine tolerance and dependence can develop in the spinal cord without neuronal influence from the brain. Functional changes in monoaminergic neurons are therefore not necessary for these behavioral changes.

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Do corticospinal fibres send collaterals to the lateral reticular nucleus?

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The inferior olive and the lateral reticular nucleus (LRN) are major sources of cerebellar afferents. Both nuclei relay information from the spinal cord and from supraspinal motor centres. It has been proposed that these nuclei function as comparators; the hypothesis assumes that the ascending spinal information relates to the function of lower motor centres which are controlled by commands from higher motor centres and that the nuclei are informed of these descending commands by collateral action from the fibres to the lower motor centres (Clendenen et al. 1974). If such collaterals exist their stimulation in the nuclei should evoke the same action as impulses in the parent fibres. We have tested this possibility with regard to corticospinal fibres and the LRN by comparing the extracellular focal synaptic potentials evoked in forelimb segments by electrical stimulation in the contralateral bulbar pyramid and in the ipsilateral LRN. Single pyramidal volleys gave a large negative focal potential in Rexed's layer VI but stimulation (200  $\mu$ A) in the LRN evoked no trace of such effect in this region. The latter stimuli gave effective activation of afferents to LRN since a large negative focal potential was evoked more ventrally in the motor nuclei due to stimulation of ascending collaterals from C3-C4 proprioceptive neurones which

have bifurcating axons projecting both to the LRN and forelimb motoneurons (Illert & Lundberg 1978).

Our results thus fail to support the suggestion that corticospinal fibres send collaterals to LRN (Langner & Wiesendanger 1973). These authors stimulated the spinal cord after degeneration of ascending fibres and pointed out that the excitation evoked in many LRN cells was due to antidromic stimulation of corticospinal fibres. It can now be suggested that these antidromic effects might have been due to stimulation of the descending branch of the above mentioned C3-C4 proprioceptive neurones which also project to the LRN.

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Effect of secretin and plasma  $K_a$  ion concentrations  
on pancreatic bicarbonate secretion

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pancreatic  $HCO_3$  secretion implies active transport of protons to interstitial fluid and is probably secondary phenomenon to such transport. Pancreatic  $HCO_3$  secretion has been proposed to be due to pancreatic proton pump; the rate of proton transport would be determined by the electrochemical proton potential gradient sustained by the proton pump (Søder et al. 1979). Hence extracellular proton concentration may also determine the rate of pancreatic  $HCO_3$  secretion. An alternative hypothesis is that pancreatic proton flux is secondary to pancreatic  $K_a$  transport (Swenson and Solomon, 1978). Both would increase the electrochemical proton potential gradient generated by  $p$  pancreatic proton pump. Increases in plasma  $K_a$  concentration would augment its dependent proton flux.

To distinguish between these possibilities experiments were performed on 11 anesthetized pigs. By infusing intravenous secretin infusion rate from  $1.6 \pm 0.2$   $\mu$ g/kg b.w/h pancreatic  $HCO_3$  secretion was raised from  $79 \pm 9$   $\mu$ mol/min to  $30 \pm 1$   $\mu$ mol/min (8 pigs). Pancreatic  $HCO_3$  secretion varied in proportion to plasma  $pH$  during infusion of plasma  $pH$  by intravenous infusion of  $HCl$  or addition of  $CO_2$  to inspired air. Calculated secretin secretion occurred at plasma  $pH$  6.86  $\pm$  0.03 and

$pH$  6.78  $\pm$  0.02 during low and high rates of intravenous secretin infusion, respectively.

During constant intravenous secretin infusion ( $2.70 \pm 0.1$   $\mu$ g/kg b.w/h) plasma  $K_a$  concentration was increased from  $144 \pm 1$   $\mu$ mol/l to  $193 \pm 2$   $\mu$ mol/l by intravenous 30% NaCl infusion (3 pigs) and lowered from  $139 \pm 1$   $\mu$ mol/l to  $113 \pm 3$   $\mu$ mol/l by intravenous  $H_2O$  infusion (3 pigs). Variations in plasma  $K_a$  concentration from  $113$  to  $193$   $\mu$ mol/l did not increase rate of pancreatic  $HCO_3$  secretion.

It is concluded that secretin increases the rate of pancreatic  $HCO_3$  secretion. This effect may be mediated by an increase in the electrochemical proton potential gradient from pancreatic cell to interstitial fluid. Increases in plasma  $K_a$  concentration from  $113$  to  $193$   $\mu$ mol/l does not augment pancreatic  $HCO_3$  secretion. These findings favour the view that pancreatic  $HCO_3$  secretion is consequence of active proton transport to interstitial fluid by pancreatic cell proton pump.

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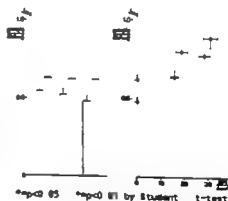
#### Physical properties and metabolic rates of respiration of muscle tissue from normal and hypertensive rat

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A study has been made of the passive and active length-tension relations, rates of  $O_2$  consumption ( $\dot{V}_{O_2}$ ) and of lactate production ( $\dot{V}_{LA}$ ) in veins of 22 wt. 10 (20-25) spontaneously hypertensive rats (SHR 313  $\pm$  5 g 88 mmHg) and age-matched normotensive Wistar-Kyoto rats (WKY 342  $\pm$  10 g 100 mmHg). Mean BP (tail artery) and anaesthesia (10% chloral hydrate) were higher in SHR. Segments of the abdominal aorta were mounted in glass chambers for registration at circumferential force  $\dot{V}_{O_2}$  and  $\dot{V}_{LA}$  (Hellstrand P. 1977 Acta physiol. scand. 100 141-143). Adjacent portions of the same aortas were removed separately for length-tension study. At rest, force/length was positive and active tension/force were higher in SHR. Optimal force/length (OC) for active tension development was lower in SHR. At OC active wall stress (force/area) was somewhat higher in SHR.

By aortic studies were performed at OC ( $P_{O_2}$  150 mmHg). Aortic rates were given relative to dry tissue weight (30-50% of wet weight) and difference between SHR and WKY. Fig. (left panel) shows  $\dot{V}_{O_2}$  and  $\dot{V}_{LA}$  in normal medium with 2.5 mM  $Ca^{2+}$  (A) and in  $Ca^{2+}$ -free normal (B) or  $Ca^{2+}$ -high (100 mM  $Ca^{2+}$ ) (C). The result shown by rose-hatched bars. The right panel shows  $\dot{V}_{O_2}$  vs active tension (P) in  $Ca^{2+}$  medium with 0.5, 2.5 and 2.5 mM  $Ca^{2+}$  (filled bars). At all levels  $\dot{V}_{O_2}$  is higher in SHR. The slope of the relation, indicating the metabolic cost of contraction, is steeper in both groups in

C10



rel. ad muscles of normal medium  $\dot{V}_{LA}$  was the same in both groups and not dependent on presence of  $Ca^{2+}$ .  $\dot{V}_{LA}$  was lower in  $Ca^{2+}$ -high solution and decreased with increasing  $[Ca^{2+}]$ . No difference between SHR and WKY was seen in normal medium  $\dot{V}_{LA}$  was 0.85  $\pm$  0.10 and in  $Ca^{2+}$ -high solution (2.5 mM  $Ca^{2+}$ ) 0.50  $\pm$  0.05  $\mu$ mol/min g (n=10 (WKY)).

In a study of portal veins no difference in  $\dot{V}_{O_2}$  or  $\dot{V}_{LA}$  was noted between SHR and WKY.

The metabolic differences found between SHR and WKY were most prominent in the basal  $\dot{V}_{O_2}$  possibly relating to increased active membrane transport. However the result may also be compatible with difference in muscle mass as fraction of tissue weight.

Skimmi g of mic sph s us s f l  
timat s of blood flow di trib tio  
i the d g kidn y  
■ CLAUSEN A KIRKES I TYSSBOTN  
K s ØFJORD & K AUKLAND  
Institut of Ph y i o l o g y Univ ity of  
B r g M r w a y

D r i g e n a l v a d i l t i o n 15 µm m i o  
ph (Ms) i d i a t t h t v a l r  
o d t a e i n r s e p p o t i o n t e l y m r e  
i i t h m i n o u t r c o t i c l o n  
Thi l d b d u = f l u r e f M t o  
n t t h i n r f f r t r t r i o l s i n  
p p o t i n t o b l o o d f l o w g g s t d b y  
p l i d s p r i o n f l t i t b l o o d  
f l w d m c o c a t t i o (C l m t l  
1979) R e n t m o d l p r i m n t (Øfj d  
t l 1979) i n d i c e t t 15 µm M s y  
h k i m m d l a n g t h i t l b l e t r y  
(i l ) s u c h k i m m g w o l d b p e c t d  
t b d p n d t o l l d i s t d t  
b l s p r o o n d f m l l e p h  
W t h s f o r i j t d a m i t u f 10 µm  
d 15 µm M f t i m a t i o n o f b l o d f l w  
i c t i l d m d i l l a r y n i b  
m = l k i d y d i 4 k i d n y w i t h  
a b o u t 50% i d s l d t o  
p d d b y i f s i f t y l b l i n e  
P i r d d t a i d i t e d t h t 15 µm M d  
e t i m t d i n t i l f l w b y b o t 20%  
a c m p r d t 10 µm M d i g c o t l d  
b y l t h 10% d r i g v d i l t i  
P v i e u l t i d i t t h t 15 µm M

u n d r t i m a t i n r t i c l f l o b y b o  
60% d e i n g = t r l e s u 15 d i g s  
d i l a t i o a c o m p d t o n l t e d o a l  
f r a t i o l f l w t i a t d f o m l i  
n t i p y i n u p t k r t e (C l s t l 197  
Thus both M s l s s e e = d r t i t  
d e p c o t i a l f l w a d t i m a t o u t  
o r t i c a l f l o i c t r o l k i d y 15 µm M  
t o g r a t r t a t t h a 10 p M s A l t h o w  
t h i u n d e r s t i m t i d e d d r i g s  
d i l a t i o n t h h g i t h 10 µm a n d 15 µm  
M s d i s t i b u t i o n m o g = b i l y g l  
f a l d d i v r g i n g t i t f h a g i  
o n l b l o o d f l o w  
S i n c e g n a l w t r t i f t h M  
v s 99 % d o n l y a i r f r a t i o f M s  
w s l m t d i n p o t g l u l p i l l e i s l  
w e n l d t h t f l t y i n t l d i t i  
b u t i f b l d f l w s t i t d b y M i  
d u e t k i m m i g o f M a l o g t h i l a A  
x p t d t h k i m m i n g p p = t b e l s t  
p r o = d w i t h t h a l l p h d t h  
l r g i l d i t

R e f e r e n c e s  
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Phy i l s d 107 69 81  
ØFJORD E s CLAUSEN G s AUKLAND K  
1979 Up l J M d s i s p p l 26 58  
(Ab t )

Reduced GFR i d hyd ted animal i the tubulo-  
glomerula feedba k  
G SELÉN & A E H PERSSON  
Dept f Ph y i l o g y & Medical Biophys i Univ ity  
f Upps l Sweden

Earli experiment have hown the exist n c f  
tubulo-glomerul s feedback control that educ  
glomerul sp l l r y p s u r e and glomerul r f i l  
t i o n r t t i n c e s s e d d i s t a l d l i v e r y f f l i d  
The s e n s i t i v i t y f t h i m e c h a n i s m c a n l b e e-  
t d u r i n g d i f f r e n t p h y s i o l o g i c a l c o n d i t i o n s C a  
p i l l r y m i r o p e f u s i o n e x p e r i m e t h a v r e v l e d  
t h a t t h e p l a s m a c l o i d c o n c e n t r a t i o n p e f u s i g  
t h e m a c l d e n s a n m o d l c f e e d b a k s e n s i t i v i t y v i a  
h a n g e i n ( t r a t i c l ) p r e u r e a n d  
v o l u m e ( P E R S S O N A E G s M Ü L L E R S U U R R s S E L É N G  
1979)

The q u e s t i o n i s a i f i d h y d t e d a n i m a l  
t h e f e e d b a c k s e n s i t i v i t y i s a s e d a n d f t h e e-  
d u c t i o n i n k i d n e y G F R o b s e r v e d i t h i o n d i o n  
c a n b e e x p l a i n e d b y a n a t i o n o f t h e t b u l o-  
g l o m e r u l f e e d b a c k c o n t r o l D e h y d r t e d a n i m a l  
(24 h r s f o o d a n d w a t ) w e r e t u d i e d b e-  
f o r e a n d a f t e a s l i n e v o l u m e e x p a n s i o n (5% f B W )  
G F R w a s f o u n d t o b e s i g n i f i c a n t l y r e d u c e d d i g  
d e h y d r t i o n c o m p e d w i t h c o n t r o l c o n d i t i o n (0 %  
d e h y d r t i o n a n d 1 4 9 0 0 9 m l / m i n r e p e c t i v e l y )  
d u r i n g v o l u m e e x p a n s i o n G F R r o s e t o 1 7 1 2 0 0 8 m l /  
m i n

I n m i r o p o s t u r e e x p e r i m e n t s t a b a l e t o p f l o w p  
s u r e w a s r e c o r d e d t d i f f r e n t r t f t i c i l i  
p e r f u s i o n f t h e d i s t a l n e p h r o n T h e f e e d b a k h a r-  
p e r f u s i o n s w e r e d e t e r m i n e d f r o m t h e m a x i m a l d o p  
a c t l e t s w e r e d e t e r m i n e d f r o m t h e t o p f l o w  
i s t o p f l o w p s u r e i p e r c e n t f t h e t o p f l o w

p s u r ( Δ S P F X ) a n d t h e p e f u s i o n = t t v a l t  
50% o f t h e m a i n a l t o p f l o w p r a s u r d r o p w a s b r-  
t l o e d t h e t u r n i n g p o i n t D u r i n g d h y d t i o n t h e r  
w a s a s i g n i f i c a n t r e d u c t i o n i n t u r n i n g p o i n t f r o m  
20 7 ± 8 1 / i n t 16 5 ± 2 0 4 1 / m i n a n d a n i s o-  
o s a i Δ S P F X f r o m 23 5 ± 1 8 t 34 2 ± 4 0 m m  
p a e d w i t h c o n t r o l c o n d i t i o n s i n d i c a t i n g a n i s o-  
o s e d f e e d b a c k s e n s i t i v i t y w h i l d u r i n g v o l u m e  
e x p a n s i o n t u r n i n g p o i n t w a s i n c r e a s e d t m o r e t h a n  
35 1 / m l a n d Δ S P F X e d u c e d t 8 0 ± 3 3 m i n t i  
d e c a s e d f e e d b a c k s e n s i t i v i t y

T d t r a l i f t h i i n c r e a s e d f e e d b a c k s e n s i t i v i t y  
d i g d h y d t i o n g a v e i n s t f e e d b a c k l a s  
e d u c t i o n i G F R d i t l a n d p r o x i m a l l l e c t i o n s f  
S E N G R A w e m a d i f t h e s a m e n e p h r o n D i s t a l c o l l e c t  
o n w i t h a n i t s f e e d b a c k l o o p g a v e s i g n i f i c a n t  
l o w e l u e t h a n o x i m a l l l e c t i o n w i t h t h e f e e d  
b a c k l o o p b r o k e n (32 2 ± 9 1 / i n a n d 37 3 ± 2 6 1 /  
m l r e s p e c t i v e l y ) i o n t l r i n o d i f f e r e n c e  
c o u l d b e d e t e c t e d T h i r e s u l t i n d i c a t e d t h a t t h e  
f e e d b a k s y s t e m i s i n v o l v e d i n r e d u c i n g G F R i d e b y  
d t e d a n i m a l

I c o n c l u d e t h a t t h e d u c t i o n i t t l k i d n e y G F R i  
o c u r s d h y d t i o n i t l t p a r t l y r e l a s e d i  
t h e t u b u l g l o m e r u l f e e d b a k m e c h a n i s m t h a t h a  
g l o m e r u l i n c r e a s e d s e n s i t i v i t y d u i g t h i s c o n d i-  
t i o n T h i s i s i n g r e e n t w i t h t h e t h e o r y  
t h a t t h e p r e u r e - v o l u m e c o n d i t i o n f t h e i t r t  
t u r r o u n d i g t h e m a i d e n s i t y m o d l t  
t h e s e n s i t i v i t y f t h e t u b u l o g l o m e r u l f e e d b a k s  
h a l i m

R e f e r e n c e s P E R S S O N A E G s M Ü L L E R S U U R R s S E L É N G  
1979 A n J Physiol 236(2) F97 F102



The effect of the situation and training on two and one leg extension at the pth

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The classic concept is that it is possible to recruit all motoneurons simultaneously when performing a maximum voluntary muscle contraction (MVC) (Morton 1954). However, recent determinations of the ratio ( $R_M$ ) between MVC of extending both legs simultaneously and MVC of the two legs working independently show that untrained subjects are unable to develop their full muscle strength during extension of both legs when they are working together (Secher 1975, Secher et al 1978). The aim of the present study is to investigate whether the low  $R_M$  value of untrained subjects is due to a lack of habituation or a lack of training.

MVC was determined with both legs working simultaneously and with each leg working alone in 18 subjects at three occasions separated by 5 weeks. The 18 subjects formed three groups: 5 in the reference group, 6 trained isometrically by performing 150 MVC 36 times with right and left leg separately and 7 subjects trained with 150 repeated MVC 16 times with both legs. At the first determination two different leg MVC were 3000

00 (SE) and 1660  $\pm$  92 N with a  $R_M$  value of  $82 \pm 2.6\%$ . At the second determination two leg MVC was 3200  $\pm$  40 N while one leg MVC had decreased to 1600  $\pm$  84 N. Thus  $R_M$  had increased to  $97 \pm 3.0\%$  irrespective of the type of training applied: two and one leg MVC increased by  $55 \pm 9.0$  and  $40 \pm 5.0\%$  resulting in a  $R_M$  value of  $101 \pm 3.3\%$ . During the training period the reference group changed two and one leg MVC by 2.0 and 14.1%.

The results suggest that the low  $R_M$  value of untrained subjects reflects a relative impairment of the motoneurons when the legs are used simultaneously but that the impairment vanishes with the situation.

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## C12

Effect of increasing glucose loads on the rate of muscle glycogen resynthesis after prolonged exercise

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Muscle glycogen declines progressively during submaximal exercise requiring 60-80% of maximal oxygen uptake ( $\dot{V}_{O_2 \max}$ ) until total depletion at exhaustion (Nerhusen et al 1967, Gollnick et al 1973).

In view of the important role that glycogen plays during prolonged exercise, resynthesis of glycogen in muscle and liver must be an important component of the recovery process.

The aim of the present investigation was to study the effect of increasing glucose loads on the rate of muscle glycogen resynthesis after prolonged exercise. Five muscle biopsies were obtained from 12 male subjects before and at various intervals during the first 8 hours of recovery after prolonged exercise (10-70% of  $\dot{V}_{O_2 \max}$  to exhaustion).

Three series of experiments were performed with 4 subjects in each group. The subjects were given 0.7, 1.4 or 2.0 grams of glucose (in water) per kg body weight every 2nd hour of the recovery period. The mean rates (SE) of muscle glycogen resynthesis were  $2.3 \pm 1.2$ ,  $4.6 \pm 0.7$  and  $5.6 \pm 1.4$  mmole glucose units

kg<sup>-1</sup> wet weight hr<sup>-1</sup> respectively. It is concluded that the rate of muscle glycogen resynthesis after prolonged exercise increases with increasing oral glucose loads up to a certain level. A glucose load above this level gave no further increase in the rate of muscle glycogen resynthesis.

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The redistribution of ACh receptor hotspots during synapse elimination

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During synapse formation throughout the nervous system it is common for an excess of synapses to be formed initially and for many of these to be subsequently eliminated. The present study used the formation of ectopic neuromuscular junctions on the adult rat soleus muscle to investigate whether elimination is random with respect to distance along the postsynaptic cell or whether, for example, a synapse is more likely to be eliminated if it is close to another synapse.

The fibular nerve was transplanted onto the soleus muscle at a site proximal to the original endplate band and allowed to grow throughout the muscle fibres for three to four weeks. The olivus (tibial) nerve was then cut and resected which caused the formation of neuro-

muscular junctions by the fibular nerve in three days (Lomo & Elter 1978). We used  $^{125}$ I labelled  $\alpha$ -bungarotoxin and autoradiography to map the location of ACh receptor hotspots induced at new synapses.

Initially the fibular nerve induced several hotspots along each muscle fibre. After 21 days about 80% of these had been eliminated. At 6 days after cutting the fibular nerve to induce synapse formation the receptor hotspots were distributed at random along the muscle fibres. The distribution of distances between adjacent hotspots followed a negative exponential curve. If the elimination process operated at random one would expect the distribution of hotspots at 21 days also to be random. However this was not the case. Therefore elimination of hotspots was not a completely random process. Rather our data support the idea that the probability that a hotspot will be eliminated depends on the distance to its nearest neighbour. LOMO T & ELTER C R 1978 J Physiol 275: 391-402  
This work was supported by the New Zealand IRC

## C16

A likely physiological role of spermatozoal zinc

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The highly S-S crosslinked mammalian sperm nuclear chromatin gives the sperm nucleus its high resistance to different chemical and physical conditions (cf Calvin & Bedford 1971). It is generally accepted that the ovum provides free thiol-enzyme systems of high efficiency to cleave the S-S bonds.

However, in human sperm nuclear decondensation has recently been performed without exogenous S-S cleaving agents (Kvist 1979). It was suggested that the human spermatozoon may have an intrinsic free thiol system with ability when triggered to cleave intermolecular S-S bonds and thereby allow a rapid nuclear decondensation upon sperm entrance into the ovum. Evidence was provided for the concept that the nuclear chromatin decondensation ability (MCD-ability) is reversibly inhibited by zinc derived from the prostatic fluid upon ejaculation. The MCD-ability could be reactivated by zinc removal induced by conditions known to induce fertilizing capacity of ejaculated spermatozoa.

The aim of the present study was to evaluate if spermatozoal zinc also would protect the sperm MCD-ability from oxidative destruction upon storage. The MCD-ability of untreated and zinc-depleted spermatozoa was examined before and after storage for 24 h in a buffered salt solution with and without addition of zinc ions. The proportion of spermatozoa with MCD-ability was significantly reduced in all groups upon 24 h storage almost totally (by 88%) in the zinc-depleted and by 44% in the untreated group.

In the zinc-depleted and zinc supplemented group the reduction was only 36% a value not differing from the untreated and zinc supplemented group (38%). Other differences significant (p < 0.05).

The results are in agreement with earlier reports (cf Kvist 1979) that human spermatozoa lose their MCD-ability upon storage an effect that also can be rapidly induced by oxidative agents, e.g.  $\text{Cu}^{2+}$ .

The results indicate that spermatozoal zinc derived from the prostatic fluid upon ejaculation might be of physiological importance to protect the sperm MCD-ability in man. Such a protection would also be manifested as a reversible inhibition of the sperm MCD-ability which in turn creates a need for its reactivation by depletion of sperm zinc in the female genital tract. In favour of these suggestions are the following observations of Chang and Austin in 1951 (cf Chang & Hunter 1975) that the spermatozoon has to remain in the female genital tract for several hours to gain its fertilizing capacity and that the spermatozoa release zinc during this period (Gunn & Gould 1958).

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The vagal control of the feline pylorus

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The pyloric motor activity was recorded by  
by the change of an externally applied flow of  
high-salt solution across the pyloric sphincter on  
distal perfusion pressure in cat chloralose  
anesthesia (Edna et al 1979). The two  
were cut unilaterally and the distal and proximal  
and respectively were inserted into the  
electrode and stimulated electrically. When the  
pylorus was given in the out direction using a  
threshold parameter (2.0 V 5 sec) 0 Hz  
according to Martenson (1965) biphasic motor  
response with an initial dilatation followed by  
myoelectric contraction was obtained. When  
the stimulation was given after closure of the  
pylorus by infusion of atropine (0.01-0.02  
mg/kg/min) dilatation was induced  
indicating that the vagus nerve both acetylcholine  
and inhibitory fibers to the pylorus. At 5 sec  
after vagus stimulation there was pyloric  
relaxation, which disappeared after division of the  
unilateral vagus nerve.

tion & Herent stimulation pe l ted unaffected  
The dilatation wa pre ated if hexamethonium wa  
followed by tropine (0.2 mg/kg) in cat with in  
tact planchni nerve The sult indi to that  
the vagu exerts both itatory and inhibitory  
ontrol of the feline pylorus via non-choline gi  
on-ad no gi nerve fibre Y ribe experiment  
have indicated that peptide ( skephalin, va  
tive into tinal peptide) may be involved in the  
vagal out ol (Edin t l 1979 b )

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## C 22

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 2. 19 1. glutamat satur 1. trans  
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 Division of Toxicology Kjeller, Norway

Several lines of evidence indicate that glutamate (GLU) may be a transmitter in the hippocampus. However, it is only partially identified since from a synaptic point of view it is responsible for physiological stimulation. We have reported that the 1 of D-glutamate (ASP) in response to electrical stimulation of two well defined axonal branches from CA3 pyramids the Schaffer collaterals and the hippocampal projection through fimbriae.

Experiment 1  
 Transverse hippocampal slices (Shelton & Swadlow 1971) and on new slices prepared with the tang fimbria As for hippocampal slices fib volleys single and double discharges and evoked potentials were recorded from the septal preparations.

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 11 ferents to dora 1 t rai septum  
 11 fish la r 1 used D-ASP in ri

mus dependent manner. The slices were  
loaded with radiolabelled amino acids  
and placed in a superfusion cell before  
stimulation at rest.

The stimulus evoked a less filled a verbal of the requirements for neuro t transmitter ide tification in as much s it wa Ca depe de t and pe lfi A presynaptic fibr volley wa present ; both high (1.5 mM) and low (0.1 mM) Ca<sup>2+</sup> but D- [40] ASP was rel s ed t high C<sup>2+</sup> only W ith GABA no L leuci w r r l ased in re pon e t stimu lation (25 Hz Hr)

However, D-ASP does not discriminate between glutamergic and dopaminergic terminals but is at inhibitory synapses from our laboratory allow for such discrimination. For example, both surgical and chemical lesion of the CA3 axon collateral were accompanied by a significant decrease in both high-affinity uptake of L-GLU and its endogenous concentration of L-GLU in lateral septum with no changes in endogenous level of L-ASP (review Fonnun et al 1979).

Thus in conclusion our results, which are consistent with Dale's principal findings, show that L-GLU and not L-ASP is the neurotransmitter of the hippocampal CA1 neurons.

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ATP per se inhibits non cholinergic non adrenergic transmission in the rat urinary bladder

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The excitatory transmission in the urinary bladder appears to be predominantly non-cholinergic non adrenergic (Ambache & Zar 1970). Since ATP contracts the detrusor muscle and since ATP may be released during nerve stimulation the atropine resistant transmission in this tissue has been considered purinergic (Burnstock et al 1978). Recently however it was shown that purines including ATP inhibited the contraction response to transmural nerve stimulation (TMS) in the guinea pig and rat urinary bladder (Dahlén 1979). The assumption that the inhibitory action of ATP is caused by metabolites eg. adenosine (Brown et al 1979) was tested here.

Rat detrusor strips were placed in organ baths containing Krebs-Henseleit's solution gassed with 5% CO<sub>2</sub> in O<sub>2</sub> and kept at 37°C. Responses to TMS (5 Hz, 10 ms suprathreshold voltage, 10 pulses at 1 min interval) or acetylcholine (ACh) were recorded isotonically (load 5 g) or isometrically (tension 5 mN). ATP (1 000 µM) and adenosine (1 000 µM) dose-dependently and reversibly inhibited both the contraction response to TMS and the contractions caused by ACh (50 µM) or direct smooth muscle activation by long pulses (5 Hz, 150 ms). Treatment of the tissue with adenosine deaminase effectively annulled the inhibition by adenosine while the inhibition by ATP remained unaltered. Furthermore the stable ATP-analogue 8-γ-M-ATP (1 000 µM) also inhibited the contraction response to TMS and ACh being at least

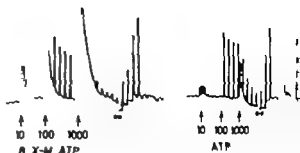


Fig 1 Effects of cumulative doses of 8-γ-M-ATP on contraction responses to TMS in the rat urinary bladder in vitro. Drugs added at arrows. Bath fluid changed at dots.

as potent as ATP (Fig 1).

These findings seem to be at variance with the concept of purinergic excitatory transmission but suggests the presence of postjunctional purine receptors that inhibit non cholinergic non-adrenergic transmission in the rat urinary bladder.

Supported by grants from the Swedish Medical Research Council (4342) and Karolinska Institutet.

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## C20

Possible role of prostanooids in the regulation of smooth muscle activity in the human ovary

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By the use of an in vitro model which permits mechanical separation of muscle layers with the aim to orient them we were able to reveal differentiation of adrenoceptor responses of smooth muscle in the isthmus portion of the human oviduct, a region which is believed to play a key role in the proper timing of ovum transport (Lindblom et al 1979). The present report deals with the possible significance of prostanooids in the control of contractile activity in the human oviduct.

Tissues from fertile women were obtained by operation. The isthmus portion of the oviduct was rapidly excised and immersed in chilled oxygenated Krebs-Ringer bicarbonate buffer. Muscle strips with cross sectional areas of approximately 1 mm<sup>2</sup> and length of about 4 mm were dissected from the uterine muscle layers, the ampullary isthmus junction (AIJ) and the utero junction (UIJ). The contractile properties of these specimens were then studied in organ bath experiments.

Addition of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) to the organ bath caused a concentration-dependent inhibition of the spontaneous phasic activity in the circular muscle layer of both the AIJ and the UIJ but induced an excitatory response in the external longitudinal layer. In the innermost longitudinal muscle layer the effect of PGE<sub>2</sub> was dependent

on the phase of the menstrual cycle: an excitatory response was constantly achieved in the periovulatory period whereas in the other phases of the cycle there was an inhibitory effect. PGE<sub>2</sub> on the other hand evoked powerful excitatory responses in all muscle layers independent of the cycle phase. Prostaglandin (PGI<sub>2</sub>) contracted the outer longitudinal muscle layer in the same manner as did PGE<sub>2</sub> and stimulated the innermost layer in all phases of the menstrual cycle. PGI<sub>2</sub> however had very weak effects on the intermediate circular layer.

Indomethacin, an inhibitor of PG synthesis, caused concentration-dependent inhibition of contractile activity in all types of muscle layers which could be reversed by the addition of small amounts of PGE<sub>2</sub>. Furthermore it was found that excitatory responses produced by the α-adrenoceptor agonist phenylephrine were unaffected by indomethacin.

In conclusion the results demonstrate the differentiated distribution of PG receptors in the smooth muscle layers of the human oviduct. Moreover the data suggest that endogenous prostanooids are prerequisites for spontaneous activity in this type of smooth muscle under in vitro conditions.

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peripheral diameter in two-  
lumen clip hypertensive rats

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The entrance of microspheres (MS) from  
arterial arterioles (AA) into glomeruli (GL)  
has been used to estimate mean diameter of  
the AA (Mikridis et al 1978). The method is  
used in the study of 8 normal rat  
kidneys (NC) 6 non-clipped (NC) and 6 clip-  
ped (CC) kidneys from 6 clip-hypertensive  
rats 70 days after clip application and  
6 similar pairs of kidneys (PC and CC)  
4 days after clip application and 12 days  
after clip removal.

The following results were obtained

	RF (ml/min)	RF (ml/min/g)	d (µm)
NC	154 ± 4	2.73 ± 0.23	19.2 ± 0.4
CC	217 ± 15	2.80 ± 0.39	16.5 ± 0.2
PC		2.55 ± 0.53	20.8 ± 0.6
CC	133 ± 13	3.19 ± 0.28	17.1 ± 0.6
CC		3.09 ± 0.40	19.1 ± 0.4

RF as mean ± SEM and the entrance  
area of MS from AA into GL from which  
RF-values have been calculated are  
shown in the figure.

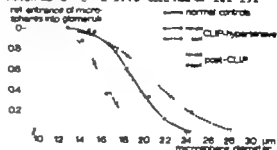
Application of the clip induced signifi-  
cant systolic arterial pressure (SAP)  
increase which in turn resulted  
in significantly increased d ( $p < 0.05$ )  
in clipped kidneys (CC) and significantly  
increased d ( $p < 0.0002$ ) on the non-clipped

kidney (NC) to maintain unchanged renal blood  
flow (RBF). Removal of the clip allowed SAP  
to return to normal values also with-  
out the return of d back to normal on  
the clipped kidney (CC). In the non-clipped  
kidneys (PC) d was not significantly larger  
than in the corresponding kidneys (NC)  
during clip-hypertension ( $p = 0.2$ ) and d  
was still significantly lower than for the  
normal control ( $p < 0.02$ ).

This indicates that perfect AA RBF auto-  
regulation is present for 70 days on the  
clipped kidney whereas hypertension induced  
dilatation of the AA in the un-  
clipped non-clipped kidneys was not abo-  
lished by normalisation of SAP for 12 days.  
Since these kidneys still have not reduced  
RBF compensatory vasodilation must have  
occurred somewhere else in the kidney.

#### R. F. KENNY

MORFID L. 1 1978 Circ Res 42 181 191



Microspheres blood flow  
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Microspheres blood flow  
1.5 ml/min/g

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The effect of barbiturate anaesthetics on motoneurone depolarizing responses to glutamate and DL homocysteate

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Intracellular recordings from cat spinal motoneurons have revealed differences in the mechanism underlying depolarizing responses to excitatory amino acid analogues (Engberg et al 1979). In this study we have investigated the interaction of barbiturate anaesthetics with the depolarizing responses of motoneurons to glutamate and DL homocysteate (DLH).

Intracellular recordings were made on motoneurons in anaemically decorticated cats. The amino acids were applied iontophoretically from extracellular barrels surrounding the recording electrode. The barbiturates pentobarbitone and thiopentone were either injected slowly (4-15 mg/kg) or applied iontophoretically. Blood pressure was carefully monitored throughout and experiments were rejected where vascular movements had clearly caused instability of the recording. Membrane input conductance ( $G_m$ ) was measured by injection of constant current pulses through the intracellular recording electrode.

On resting neurones 1 V doses of barbiturates decreased spontaneous synaptic activity and caused a 2-4 mV hyperpolarization with no change in  $G_m$ . Long lasting glutamate-evoked depolarizations (accompanied by a slowly increasing  $G_m$ ) were

usually decreased in size by the barbiturates though occasionally increases in response size were seen. In both cases however the barbiturate reversed the large increase in  $G_m$  evoked by glutamate.

DLH evoked depolarizations (accompanied by a stable decrease in  $G_m$ ) were always attenuated by the barbiturates. The depression of response size was greater than that seen for glutamate when the two agonists were tested consecutively. The barbiturates however caused no change in the conductance mechanism underlying the response to DLH.

We have previously suggested that the high  $G_m$  state evoked by glutamate may reflect low affinity glutamate uptake (Engberg et al 1979). Were barbiturates to depress this uptake this would explain the barbiturate induced reversal of the high  $G_m$  state.

Reference

ENGBERG I FLATHAN J A & LAMBERT J D C 1979  
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## C24

Spontaneous changes in membrane properties of Purkinje cells

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In recent experiments it was found that the cell membrane of Purkinje cells has pacemaker properties (Hounsgaard 1979). The aim of the study presented here was to investigate the intrinsic adaptive and slow oscillatory changes in the membrane properties of Purkinje cells.

Intracellular record ngs were obtained from Purkinje cells in cerebellar sections prepared and maintained in vitro as described previously (Yamamoto 1973).

Approximately half of the Purkinje cells were active only intermittently. Silent periods occurred at regular intervals and were initiated by a sudden spontaneous hyperpolarization of more than 5 mV. During the silent period the membrane potential and the membrane conductance decreased in parallel.  $TTX$  ( $10^{-6}$  M) reduced the amplitude and increased the duration of recorded action potentials but did not block the slow oscillations in membrane potential. Substituting  $Ca^{2+}$  with  $Mg^{2+}$  increased the amplitude of the slow change in membrane potential and obscured the normal spike generating mechanisms.

The cell membrane adapted slowly to an

injected current. The firing rate increased gradually during a depolarizing pulse and reached a maximum after more than 1 s. A preceding hyperpolarizing pulse slowed the increase in firing rate during the depolarizing pulse. This effect was correlated with the duration of the hyperpolarizing pulse in the time range of 0.1 s to 2 s.

The results show that the membrane properties of a Purkinje cell at a given time is influenced by the membrane potential during the preceding seconds. This indicates that synaptic inputs are integrated over times as long as several seconds in Purkinje cells. In addition the effect of synaptic inputs may be modulated by intrinsic oscillations in membrane properties.

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#### R fe

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Developm t l aspe ts f pulmonary lipolyti  
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E HIETANEN J HARTIALA & M SIMERO

Deps tme ts f Phy i l gy Oive sities f Tr rk  
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Ramosh 1973)

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1976 C cul ion Re 39 149-152

Changes in arterial blood flow measured  
preparatory is known by new bidirectional  
p. ad. ultrasound doppler blood velocitymeter

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University of Oslo, Norway

A new bidirectional ultrasound doppler blood  
velocitymeter has been constructed (Matteland  
1979). The instrument operates  
switchable in two frequencies (1.5 MHz and 6  
MHz) and switchable in pulsed or continuous mode.  
Four filters have been incorporated in the  
instrument in order to eliminate doppler signals  
from wall movements. A heterodyne output is  
generated by mixing the two quadrature components  
and an adjustable baseline frequency.

In our experimental setup the doppler  
instrument is interfaced to cassette tape  
recorder and to microprocessor system. The  
computer is programmed to calculate spectra every  
100 ms, instantaneous mean velocities from the  
spectra, time averages for each cardiac cycle of  
the mean velocities, maximum and minimum  
velocities, etc (Will 1977). As long as the  
cross-sectional area of the vessel is constant  
the mean velocity is proportional to flow.

The instrument has been used to measure  
changes in arterial blood flow to the brain  
(Jensen and Helle 1979), to skin areas to the  
digits, and to the testes.

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## D2

Effect of sprouting of presynaptic nerve  
terminals by lectin stimulation of the post  
synaptic membrane on partial denervation

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Norway

We have recently shown that in the guinea-pig  
superior cervical ganglion the rate of sprouting  
of residual presynaptic axons after partial  
denervation is increased by period pre-  
synaptic stimulation at the time of partial  
denervation (Henri & Kjeller 1979). The mechanism  
of this effect is unknown. In principle the  
effect of presynaptic stimulation is to be  
caused by activity there is the presynaptic end  
of the axon, the axon on tel. on both. The  
purpose of the present work was to examine  
whether presynaptic stimulation has any  
effects on the rate of sprouting.

Under pentobarbital anaesthesia the inferior  
sympathetic nerve which largely free of  
preganglionic axons was stimulated with 5 pulses  
at 20 Hz every 25 sec for immediately  
after partial denervation. The peripheral  
nerve was cut absorbed during stimulation  
was allowed to the region supplied by this nerve  
for 4 days the ganglion was removed for intra-  
cellular recording. Neurons with axons in the  
inferior postganglionic nerve were identified by  
retrograde stimulation, and the number of  
residual presynaptic axons contributing axons  
to each neuron was judged by graded  
stimulation of the presynaptic nerve. There

was an increase in the number of axons in the  
sympathetic response in neurons whose axons had  
been stimulated during the initial operation  
both compared to sham stimulated neurons in  
partially denervated control ganglia (the inter-  
polated axons increased from 3.1 to 4.0, the  
typical value in normal ganglia is 2.3) and  
compared to unstimulated neurons whose axons  
emerged in the superior postganglionic nerve in  
the same ganglion (p < 0.001, Wilcoxon, van Elteren  
test).

As in normal ganglia less than 1% of the  
neurons showed synaptic potential upon anti-  
dromic stimulation of the axons in the inferior  
postganglionic nerve. Therefore proliferation  
of collateral connections between ganglion cells  
did probably not occur.

We interpret the increase in the number of  
synaptic inputs to ganglion cell as evidence for  
sprouting of residual presynaptic nerve  
terminals. Thus period hyperactivity in  
sympathetic ganglion cell appears to have  
retrograde trophic influence on residual pre-  
ganglionic axons after partial denervation.

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## Substance P release and effects in the eye

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University of Uppsala Department of Pharmacology  
Karolinska Institutet Stockholm and Department of  
Physiology Faculty of Veterinary Medicine Swedish  
University of Agricultural Sciences Uppsala  
Sweden

The undecapeptide substance P (SP) discovered by  
v Euler and Gaddum and widely distributed in the  
nervous system has been proposed as a sensory  
transmitter. It has been suggested that SP release  
from peripheral nerve endings causes the vasodi-  
lation and permeability changes that characterize  
axon reflexes (see Lembeck 1953).

In the eye at least two mechanisms seem to be  
involved in the response to various irritants: one  
involves the prostaglandin system, the other a  
neural component.

In a recent study (Bill et al. 1979) we found that  
both electrical and mechanical stimulation of the  
trigeminal nerve in albino rabbits distal to the  
Gasserian ganglion caused release of a SP-like  
immunoreactive agent (SPLI). Both nerve stimula-  
tion and local injection of SP (0.1 or 1.0 µg) caused  
myosis, a rise of the intraocular pressure (IOP) and  
a breakdown of the blood aqueous barrier. Indo-  
methacin modified but did not prevent these respon-  
ses. The effects of nerve stimulation were rather  
variable.

We have now found that stimulation of the nerve  
close to the ganglion and of the ganglion itself  
gives more reproducible results in rabbits and that  
SP has effects that seem to vary between species.

In the cynomolgus monkey cat and guinea pig  
1.0-40 µg SP injected into the anterior chamber  
had little or no effect on the pupil size, while  
1.0 µg SP produced an intense and prompt pupil  
constriction in albino rats.

Repeated mechanical stimulation of the nerve  
and the ganglion for 10 or 20 min resulted in high  
SPLI concentrations (174-666 pg/ml) of the ipil-  
teral aqueous humor (15-68 pg/ml) of the con-  
lateral and (30-106 pg/ml) of the plasma. The  
volumes obtained from the eyes varied between 40-  
150 µl. Equal SPLI concentrations were obtained  
10 and 20 min stimulation.

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## C32

Stiffness of the frog hind limb late in period  
of gl muscle fibre isolated from the frog

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The fast tension response following the stimulation of  
vertebrate skeletal muscle fibre, the latency relaxation  
(LR) which is the small reduction of the rest tension  
prior to the development of the contractile force. During  
activity muscle becomes considerably stiff, the time  
(Buchthal 1942). The increase could be associated with  
the attachment of cross-bridges to the filament.  
H. S. Igroy & H. S.ley (1973) have found evidence for  
movement of cross-bridges toward the filament during  
contraction. The increase in stiffness begins about  
the same moment the LR (H. S.ley 1950). The present  
investigation the purpose was to look into the time of  
increase more precisely. The stiffness was measured by  
subjecting the fibre to a small (0.3 mm half sarcomere)  
rapid length change before and after various times after  
stimulation. As the time resolution was limited by the  
rise time of the step (0.4-0.6 ms), the experiment were  
performed at the temperature range 3-15°C, with the  
time course of the LR was sufficiently low for fibre  
held at sarcomere length 5.3 µm and at temperature  
of 10°C the first change in stiffness occurred 5.5 ms  
after stimulation and 1.5 ms after the onset of the LR.  
The stiffness increased as time went on, and  
when the tension trace was crossing the rest tension on  
level 1 the stiffness had increased 45% over the time  
of level 1. At all temperatures studied the stiffness increased  
at a temperature of 10°C or lower there was a delay  
between the time of increase in stiffness and the time of

which the rate of tension drop in the fibre decreased corre-  
sponding to 1.5-3 ms. At temperature of 14-15°C, such  
a delay could be detected and the time of increase  
at stiffness and the time of tension drop showed the same  
dependence on sarcomere length. Based on the temperature  
dependence of the time course of the LR (H. S.ley 1979),  
it was argued that the production of the tension drop  
depended on cross-bridges not the one of overlap  
between the thick and the thin filament. This coincidence  
of the sarcomere length between the time of the event  
and the increase in stiffness indicates that both  
depend on the same process and might be due to the  
movement of cross-bridges. The delay between the onset of the  
LR and the increase in stiffness indicates that the LR is  
not dependent on cross-bridges. The present results are com-  
patible with the hypothesis of H. S.ley & S. N. Knudsen (1974).  
The delay in low temperature between the onset of the LR  
and the increase in stiffness in the rate of tension drop  
makes the delay compatible with the attachment of cross-bridges  
and force generation on the plateau of two steps in steps

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# 7. Pathogenesis of deoxyglucose during ischemia in the eye

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Accumulation of deoxyglucose is the most useful indicator of metabolic activity especially in nervous tissue (Spilko et al 1977). This is so because deoxyglucose accompanies glucose into the cells where it is phosphorylated but not metabolized further. One complication in this method is insufficient  $O_2$  supply. When this occurs the cell is likely to rely on glycolysis whereby large amounts of glucose and consequently deoxyglucose have to enter the cells. The tissue will therefore show increased deoxyglucose accumulation via the actual entry turnover is likely to be depressed (Falkowski & Duffy 1979).

To investigate the possible usefulness of this phenomenon for detection of discrete regions of ischemia under perfusion we have tried to map the glucose consumption of the distal part of the optic nerve under conditions of ocular ischemia caused by elevated intraocular pressure. The idea here is to determine if and to which extent ischemic results in increased uptake in the region adjacent to perfused tissue. It has been shown previously that in the lamina cribrosa close to its anterior border there is a sharp boundary between perfused and nonperfused regions (Sjöström & Bill 1978).

In a monkey which had a normal intraocular

pressure in one eye and a pressure causing ocular ischemia in the other C14-deoxyglucose was given i.v. After 40 min the monkey was killed and the eyes removed, frozen and sliced for autoradiography. The autoradiograph showed a strong accumulation of activity in the region of the lamina cribrosa in the ischemic eye. To document details the autoradiographs and slices were studied with the aid of film scanner (microdensitometer) with 25  $\mu$ m resolution. Comparison indicated that deoxyglucose accumulation was most marked in the interior of the lamina cribrosa falling off steeply in the direction of the interior of the eye. The region of increased accumulation was ca 150-200  $\mu$ m wide. This pattern seems compatible with conversion to glycolytic metabolism in the ischemic tissue.

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## D6

# Effect of dietary calcium (Ca) on secretion of parathyroid hormone (PTH) in rats with parathyroidectomy

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Low Ca is a potent regulator of PTH secretion. PTH-secreting cells have preserved the ability to respond to acute serum Ca variations and changes in hormone secretion. We have examined if chronic changes in dietary Ca may alter secretion of PTH from rat PTH-tumors transplanted beneath the kidney capsule in young M/Rij rats (Ca-100). The animals were given standard diet 1 week following transplantation. When the experiment started the rats were divided in 3 groups which received varying amounts of Ca: low (0.09% Ca diet (0.40% Ca) and high (1.80% Ca) for 34 weeks.

After 6 weeks serum Ca was significantly different between the low and high dietary groups: 1.1-4.11 mmol/l and 2.41-0.19 mmol/l ( $p < 0.03$ ) respectively. In contrast, parathyroid concentration showed reciprocal changes: low Ca diet 1.75-0.64 nmol/l and high Ca diet 0.13-0.04 nmol/l ( $p < 0.01$ ). No alterations in serum proteins were observed during the experiment.

Serum levels of PTH were measured radioimmunologically using antisera to human PTH directed against the C and N-terminal end of the molecule. Serum was collected from week 12-28 after start of

the experiment, but increased steeply (about 4-fold) thereafter. No difference was measured in serum PTH between the 3 groups at any time point.

Gastrin is known to stimulate secretion of PTH which, on the other hand is able to suppress gastrin release. Serum gastrin was therefore measured radioimmunologically in the 3 groups. The rats on medium and low Ca diet showed lower gastrin levels than the groups on high Ca diet after 6 weeks before changes in PTH occurred. After 28 weeks the high Ca diet rats with raised serum Ca and gastrin values showed an inhibition in the gastrin levels approaching the other groups. At the time of increased serum PTH (week 28-34) the serum gastrin concentrations were low and equal in the 3 groups.

In conclusion, different dietary regimens resulted in corresponding variations in serum Ca and inverse changes in serum gastrin. Since no differences were detected in the serum PTH levels between groups on high medium and low Ca diet, secretion of the hormone and probably growth of tumour cells were not affected by chronic changes in dietary Ca. Increased serum Ca stimulates gastrin secretion and is able to sustain the inhibitory effect of PTH on serum gastrin.



Early effects of cervical sympathetic stimulation on cerebral, ocular and cochlear blood flow

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The cerebral blood vessels, the extraocular part of the retinal arteries, the blood vessels of the optic nerve, the choroid and the cochlea are supplied with sympathetic vasomotor nerves. The physiological role of these nerves has been debated upon for a long time (see Lassen 1974). During normotension electrical stimulation of the cervical sympathetic chain has little effect on cerebral flow as determined with labelled microspheres (Alm & Bill 1973). But autoregulatory mechanisms can be expected to modify effects of vasomotor nerve stimulation in many tissues. We have tried to reveal an early marked but transient effect of cervical sympathetic stimulation on cerebral, retinal and cochlear blood flow.

The experiments were performed in anesthetized rabbits. The labelled microsphere method was used to determine regional blood flow after 15, 25 sec and 5 min of electrical stimulation of the cervical sympathetic chain. At a frequency of 1 Hz there was a 5% reduction in cerebral blood flow at 15, 25 sec and 7% at 5 min. In the choroid plexus the mean reduction was 22% at 15, 25 sec but decreased to 10% after 5 min. In the cerebellum, the optic nerve and retina sympathetic stimulation had no appreciable effect on the blood flow. In the iris and the cochlea the blood flow reduction was 32 and 25% respectively on both

occasions. In the choroid vasoconstriction increased with time. In the masseter muscle there was a decrease.

No indication was thus found in the present experiment for an autoregulatory escape phenomenon in the brain, the eye or the cochlea. Some escape was noted in the masseter muscle.

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#### D4

Facial nerve stimulation and regional blood flow during hypotension

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Morphological evidence for cholinergic nerve supply to the cerebral blood vessels has existed for many years (Chorobski & Penfield 1932). The nerves are supposed to be distributed mainly via the VIIth cranial nerve, although the IIIrd, IXth and Xth cannot be excluded.

A recent study in dogs had indicated a cerebral vasodilation during stimulation of the facial nerve (D'Alecy and Rose 1977), but a microsphere study in our laboratory in normotensive rabbits did not reveal any significant effect (Stjernschantz & Bill 1979). It seemed possible that autoregulatory mechanisms normally abolish effects of vasodilating nerves.

In this study a hypotension-model was tested which might reveal normally masked vasodilatory mechanisms.

Rabbits (n=14) anesthetized with flumazenil + fentanyl (Hypnorm®) and ketamine-chloride (Ketalar®) were used. The facial nerve on one side was exposed at the internal acoustic pore; a lateral or suboccipital approach was used. The blood pressure was controlled by bleeding into a reservoir from a femoral artery. Tubocurarine (0.5 mg/kg b.w.) was used for muscle relaxation. Labelled 15 µm spheres were used to determine regional blood flow twice during each experiment according to the reference flow method.

In one group of animals the first isotope injection was carried out at arterial blood pressures between 35-50 mm H<sub>2</sub>O, and the second injection during stimulation of the nerve on one side at the same low blood pressure.

In another group of animals the nerve on one side was sectioned to stop centrifugal impulses. The first isotope was injected at spontaneous blood pressure (85-110 mm Hg) and the second injection after a pressure decrease to 35-50 mm H<sub>2</sub>O.

The results indicate a strong vasodilatory effect of facial nerve stimulation in the mandibular gland, in the mucous membrane of the tongue, in the choroid and the ciliary processes, but no effect in the brain. Sectioning of the nerve did not change the blood flow.

Thus in rabbits under general anesthesia and arterial hypotension caused by bleeding, cerebral vasodilation is not likely to be due to facial nerve activity.

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Storage of frogs at low temperature for prolonged periods induces major ultrastructural alterations of the mesenteric capillaries

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The ultrastructure and permeability of single mesenteric capillaries in the frog (*Rana temporaria*) are studied at our laboratory. Prior to the experiments the animals are kept at 4°C for varying periods of time. During storage at this low temperature the animals are starved and undergo seasonal cycles which imply major physiological alterations (Barker Jørgensen et al. 1978). The possibility that this cold storage may influence the microvasculature was investigated in a quantitative ultrastructural study. Mesenteric capillaries from two sets of frogs captured in late summer were compared. One group of animals was kept at 4°C for less than a week (short-term frogs) and the other group at 4°C for 6-8 months (long term frogs).

The mesenteries were exposed and initially fixed by superfusion with a buffered solution of aldehydes. Randomly sampled cross sections of 31 capillaries from short-term frogs and 51 capillaries from long term frogs were included in the study. Analysis of the electron micrographs was performed at a final magnification of 51600 x.

The diameter of the capillaries, the average thickness of the endothelial cells, the depth of the interendothelial clefts, and the appearance of the endothelial junctions were similar in the two groups of animals. However, the volume density of the endothelial vesicles was 12.8% in long term frogs versus 4% in short term frogs. The length of the interendothelial clefts per  $\mu\text{m}$  capillary wall was 2120 Å/ $\mu\text{m}$  in long term frogs and 1200 Å/ $\mu\text{m}$  in short term frogs. For both parameters there was a significant difference ( $p < 0.005$ ) between the two groups. In 6 out of the 31 capillary cross sections from the short term frogs interendothelial gaps 200-500 nm wide were observed. No gaps were encountered in all 51 capillary cross sections from the long term frogs.

We conclude that storage of frogs at low temperature for long periods of time induces major ultrastructural alterations of the capillaries. We suggest that the stage of the animals within the seasonal cycles and their nutritional condition should be well defined in physiological investigations of frog capillaries.

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to that of drug metabolizing enzymes by chlorinated phenols in vitro

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Chlorinated phenols were added to in vitro incubations were shown to be strong inhibitors of cytochrome P-450 catalyzed hydroxylation or glucuronidation reactions in hepatic microsomes of the rat.

IC<sub>50</sub> of pentachlorophenol (PCP) 1 the incubation decreased the activities of arylhydroxylase and ethoxycoumarin deethylase to 1/10 of the control value. 10 µM of PCP decreased the glucuronidation of 2-methoxyphenol by 50%. 1 µM of PCP was sufficient to reduce the glucuronidation of 4-methylumbelliferone to 1/10 of the control activity.

Hydroxylation and glucuronidation reactions were inhibited by tetrachloro- and dichlorophenols. The inhibitory potency was dependent on the position of chlorine substituents. At strongest inhibition of drug metabolizing enzymes by tetrachloro- and trichlorophenol was of the same order

of magnitude than the inhibition by PCP.

Phenol and chlorinated benzenes (di- to hexachlorobenzene) themselves were not inhibitors of drug metabolizing enzymes. Thus the inhibition caused by chlorinated phenols is a combined effect of both the phenol group and the chlorine substituents of the molecule.

Pentachloroaniline lightly inhibited hydroxylation reactions whereas pentachlorotoluene had no effect on the activities of drug metabolizing enzymes in vitro.

Epoxyde hydratase and glutathione S transferase activities were not affected by chlorinated phenol in vitro.

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## D14

Effect of 7-oxa-13-prostanoic acid  
on the release of LH

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Prostaglandins (PG) seem to play a role in the neuroendocrine regulation of pituitary gonadotropin secretion by stimulating the release of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus. This activity of PG may depend on their capacity to activate specific binding sites in the brain. The present study was undertaken to investigate this hypothesis by testing the PG-analogue 7-oxa-13-prostanoic acid (7-OPA). It is believed to interfere with receptor binding of PG.

The analogue was infused into the retroperitoneal space of anesthetized male rats (300 g) into intraventricular administration of PGE<sub>2</sub>, PGE<sub>1</sub>, LHRH and a concentration of 10 µg in the plasma was determined during 90 min period. Infusion of 7-OPA alone had no effect on the release of LH. However, when 44 µg of 7-OPA was infused in combination with 2 µg of PGE<sub>2</sub>, the LH releasing activity of PGE<sub>2</sub> increased with increasing primary doses of 7-OPA. At 60-90 µg the LH concentration was 2-3 fold higher in animals pretreated with 88 µg of 7-OPA

than in control rats receiving the 7-OPA diluent. The minimal effective dose of PGE<sub>2</sub> (1-0.5 µg) decreased after pretreatment of the animals with 7-OPA and formerly subthreshold dose (0.01 µg) was found to become an effective stimulus for LH release when administered subsequent to infusion of 132 µg 7-OPA. When 7-OPA (132 µg) was infused prior to PGE<sub>2</sub>, the dose-response curve of PGE<sub>2</sub> (over the range 0.01-20 µg) was displaced to the left without change in the maximal response. Administration of 132 µg of 7-OPA before an intraventricular infusion of 50 µg LHRH did not alter the stimulatory effect of this hormone on the release of LH indicating that 7-OPA acts at a suprapituitary site to potentiate the LH releasing activity of PGE<sub>2</sub>. In contrast to its potentiating effect on PGE<sub>2</sub>, induced release of LH 7-OPA did not influence the stimulatory action of PGE<sub>2</sub> (2 µg) on the secretion of LH. The present findings are consistent with the view that PGE<sub>2</sub> activates specific binding sites in the brain as an event prior to stimulation of LHRH (and LH) release. The lack of effect of 7-OPA on PGE<sub>2</sub> induced LH release suggests the existence of separate binding sites for PGE<sub>2</sub> and PGE<sub>1</sub>.

# The effect of vanadate on liver hemodynamics and bile production of the perfused rat liver

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Vanadate has recently been found to be a potent inhibitor of Na-K ATPase (Josephson & Cantley 1977) and in experiments on cats vanadate also appeared to be a potent constrictor of renal blood vessels (Larsen et al 1979). In the present experiments the effect of vanadate on liver hemodynamics and bile production was examined in 11 perfused rat livers. Furthermore the effect of vanadate on the Na-K ATPase activity in isolated canalliculi-enriched liver plasma membranes (LPM) was examined.

Vanadate caused a dose dependent fall in portal blood flow and conductance which was reduced 11.29 and 50% at vanadate concentrations of 9.18 and 50  $\mu$ M respectively. At vanadate concentrations of 9 and 18  $\mu$ M the hepatic oxygen consumption and bile flow remained constant but at 50  $\mu$ M oxygen consumption decreased 20% and bile flow 28%. The biliary concentrations of vanadate were identical with plasma vanadate concentrations. Na-K ATPase activity in LPM was inhibited 50% by 0.25  $\mu$ M vanadate.

The present results demonstrate that vanadate is a potent constrictor of liver

vasculature at concentrations which do not affect oxygen uptake or bile production. The observed decrease in bile production at high concentrations may be secondary to hypoxia and a specific effect of vanadate on bile production which to some extent may be dependent on the activity of Na-K ATPase could not be demonstrated.

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LARSEN J A THOMSEN O O & HANSEN O  
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# The distribution and function of the sympathetic innervation of liver and endocrine pancreas

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Sympathetic noradrenergic nerves with their well known cardiovascular effects have recently been found to influence several metabolic and hormone-releasing processes. Thus experiments on animals have shown a considerable influence of sympathetic stimulation on liver function. Stimulation of the liver branches of the splanchnic nerve are associated with variations in the rate at which glucose is released from the liver (Edwards 1972). In addition it has been demonstrated that stimulation of sympathetic pancreatic nerves evokes a pronounced liberation of glucagon as well as an inhibition of insulin release from the endocrine pancreas.

We have recently investigated the morphology of the sympathetic nerves to the liver and endocrine pancreas in man. With the Falck Hillarp histofluorescence techniques for visualization of catecholamines on the cellular level it was demonstrated that the human liver parenchyma as well as the human islets receive an abundant supply of nerve fibers displaying the typical catecholamine fluorescence (Nobin et al 1978). Quantitative determinations revealed that the noradrenaline content of the human liver is about 1  $\mu$ g/g wet weight which leaves little doubt that these liver nerves contain noradrenaline. Electron microscopy confirmed that the adrenergic nerve terminals contacted the individual hepatocytes. When

peroperatively stimulating the sympathetic liver nerves clearcut increase in the arterial plasma glucose concentration was achieved reaching a significant peak increase of about 2 mM above the control level at the end of 10 minutes.

Regarding the function of the direct adrenergic fibers to the islets of Langerhans we have shown that they are involved in reflex hyperglucagonemia and hypotension in response to a blood pressure drop and also that they participate in the regulation of glucagon and insulin during hemorrhage and hemorrhagic shock (Järehult et al 1979).

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## D13

Inhibition of drug metabolizing enzymes by chlorinated phenol *in vitro*

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Chlorinated phenols when added to *in vitro* incubations were shown to be strong inhibitors of enzymes catalyzing hydroxylation or glucuronidation reactions. In hepatic microsomes of the rat 100  $\mu$ M of pentachlorophenol (PCP) in the incubation mixture decreased the activities of arylhydrocarbon hydroxylase and ethoxycoumarin *O*-deethylase to 44% of the control. The 10  $\mu$ M of PCP decreased the glucuronidation of 2-aminophenol by 88% whereas 1  $\mu$ M of PCP was sufficient to reduce the glucuronidation of 4-methylumbelliferone to 47% of the uninhibited activity.

Hydroxylation and glucuronidation reactions were inhibited by tetrachloro- and dichlorophenols too. The inhibitory potency was dependent on the position of chlorine substituents. At the highest the inhibition of drug metabolizing enzymes by tetrachloro- and trichlorophenol was of the same order

of magnitude than the inhibition by PCP.

Phenol and chlorinated benzenes (di- to hexachlorobenzenes) themselves were not inhibitors of drug metabolizing enzymes. Thus the inhibition caused by chlorinated phenols is combined effect of both the phenol group and the chlorine substituents of the molecule.

Pentachloroaniline slightly inhibited hydroxylation reactions whereas pentachlorotoluene had no effect on the activities of drug metabolizing enzymes *in vitro*.

Epoxyde hydratase and glutathione S-transferase activities were not affected by chlorinated phenols *in vitro*.

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## D14

Effect of 7-oxa-13 prostanoic acid  
prostaglandin induced LH release in  
male rats

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Prostaglandins (PG) seem to play a role in the neuroendocrine regulation of pituitary gonadotropin secretion by stimulating the release of luteinizing hormone releasing hormone (LHRH) from the hypothalamus. The activity of PG may depend on their capacity to bind to specific binding sites in the brain. The present study was undertaken to investigate this hypothesis by utilizing the PG-analogue 7-oxa-13-prostanoic acid (7-OXA) which is believed to interfere with receptorbinding of PG. The analogue was infused into the cerebroventricular system of anesthetized male rats prior to intraventricular administration of  $PGF_2$ ,  $PGF_1$ , LHRH and the concentration of LH in arterial plasma was determined during 90 min period. Infusion of 7-OXA alone had no effect on the release of LH. However, when 44  $\mu$ g of 7-OXA was infused in combination with 2  $\mu$ g of  $PGF_2$ , the LH releasing activity of  $PGF_2$  increased with increasing priming dose of 7-OXA. At 40-90  $\mu$ M the LH concentration was 2-3 fold higher in animals pretreated with 88-132  $\mu$ g of 7-OXA

than in control rats receiving the 7-OXA dilution. The minimal effective dose of  $PGF_2$  (1-0.5  $\mu$ g) decreased after pretreatment of the animals with 7-OXA. At a formerly subthreshold dose (0.1  $\mu$ g) we found to become an effective stimulus of LH release when administered subsequent to infusion of 132  $\mu$ g 7-OXA. When 7-OXA (132  $\mu$ g) was infused prior to  $PGF_2$  the dose-response curve for  $PGF_2$  (over the range 1-20  $\mu$ g) was displaced to the left without change in the maximal response. Administration of 132  $\mu$ g of 7-OXA before an intraventricular infusion of 30 ng LHRH did not alter the stimulatory effect of this hormone on the release of LH indicating that 7-OXA acts at a suprapituitary site to potentiate the LH releasing activity of  $PGF_2$ . In contrast to its potentiating effect on  $PGF_2$ , induced release of LH 7-OXA did not influence the stimulatory effect of  $PGF_2$  (2  $\mu$ g) on the secretion of LH. The present findings are consistent with the view that  $PGF_2$  activates specific binding sites in the brain as a prerequisite to stimulation of LHRH (and LH) release. The lack of effect of 7-OXA on  $PGF_2$  induced LH release suggests the existence of separate binding sites for  $PGF_2$  and  $PGF_1$ .

Synaptic long lasting potentiation in hippocampus its relation to afferent fibre threshold and tetanization strength

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Guinea pig hippocampal slices *in vitro* (Skrede & Westgaard 1971) were used to study long-lasting potentiation (LLP) of synaptic transmission to CA1 pyramidal cells. Since previous studies employing two step rate inputs to the pyramidal cells (Andersen et al 1977, Lynch et al 1977) have shown LLP to be specific for the tetanized path, we now wished to determine whether the specificity holds also for different fibres within the same path.

Electric stimulation was delivered to the fibres in stratum oriens at a low repetition rate interrupted by short periods of tetanic stimulation. The slope of the relation between presynaptic volley and field EPSP was used to estimate the LLP contributed by oriens fibres with different thresholds for electric stimulation.

In 6 out of 12 slices there was a considerable contribution to the potentiation from not only the fibres recruited below tetanization strength, but also from the higher threshold fibres. This finding seems to indicate that LLP is not strictly specific to the individual fibres subjected to tetanization.

Alternative interpretations of the results are possible. For instance the high threshold

fibres would certainly contribute to LLP if they took part in tetanization, due to a temporary decrease in threshold. This seems unlikely however since no increase in the presynaptic volley amplitude as seen during the tetanus. Another possibility would be that the activation of potentiated synapses could modify transmission at those synapses which have not been potentiated.

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#### D 16

The effect of controlled physical activity on lipid metabolism in man. A longitudinal study

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Physical activity has been suggested to have beneficial effect on lipid metabolism. Most studies concerning the effect of physical activity on plasma lipids (eg Lehtonen & Viikari 1978) or postheparin plasma (PHP) and lipoproteins (Lipoprotein in lipids (LPL) (eg Nikkila et al 1978, Peltonen et al 1979) have been carried out by measuring these parameters in a group of subjects differing in their physical activity. The effect of the present study was to investigate both plasma lipids and PHP and lipoproteins in LPL longitudinally in subjects undergoing controlled physical activity program.

The training group consisted of 20 previously untrained men who trained 3 times a week for 15 weeks at least three hours a week. During the training period the control group of even age and the same period control group of even age were asked to maintain their normal physical activity. Control possible a small alterations in the measured parameters. At the beginning of the training period the groups did not differ in relation to age, relative body weight, physical fitness.

During the test period physical fitness increased significantly ( $P < 0.01$ ) in the training group, calculated by the paired t-test, while no change occurred in the control group. The training group had PHP LPL ( $P < 0.01$ ) adipose tissue LPL ( $P < 0.05$ ) HDL cholesterol ( $P < 0.05$ ) and HDL cholesterol to total cholesterol ratio ( $P < 0.01$ ) increased where total cholesterol ( $P < 0.10$ ) LDL cholesterol (estimated by calculation) ( $P < 0.02$ ) and total cholesterol ( $P < 0.05$ ) decreased during the training. No significant change was observed in the postheparin plasma lipids in the control group. The relative change in HDL cholesterol to total cholesterol ratio parallelly with the absolute change in physical fitness ( $P < 0.05$ ) and with the total change in PHP LPL activity ( $P < 0.05$ ). Our results are in good agreement with the results of other observational studies and imply that moderate physical activity program affords enough compliance benefit in changing lipid metabolism.

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The effect of adrenergic blocking agents on the release of 5 HT from enterochromaffin cells in the cat

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Cat splanchnic nerves were stimulated electrically (8-10 V; 2 ms; 10 Hz; 3-5 min) at the preganglionic level in different directions in chloralose anesthetized cats (n=6) with the adrenal lig tied bilaterally. A significant decrease ( $p < 0.05$ ) of the intra-alkala-se-aton (5 HT) levels in postganglionic ( $n=20$ ) of individual enterochromaffin cells (EC) in the mucosa from 3 different levels of the small intestine (duodenum, midjejunum, terminal ileum) was obtained. The intracellular 5 HT levels before and after stimulation were studied cytofluorimetrically in hypose-treated according to the Falck-Hillarp technique (Ahlman et al 1976).

One group of cats (n=5) was pretreated with propranolol (2 mg/kg) prior to stimulation. This prevented the decrease in fluorescence intensity. Selectively two other groups of cats (n=4 each) were pretreated with phentolamine (5 mg/kg) or phentolamine (5 mg/kg) respectively which also blocked the decrease in fluorescence intensity. No significant change in fluorescence intensity after nerve stimulation in the group pretreated with phentolamine respectively even though the drug themselves had no effect on the fluorescence intensity in control animals.

One group of cats (n=5) was pretreated with propranolol (2 mg/kg) prior to stimulation. This prevented the decrease in fluorescence intensity. Selectively two other groups of cats (n=4 each) were pretreated with phentolamine (5 mg/kg) or phentolamine (5 mg/kg) respectively which also blocked the decrease in fluorescence intensity. No significant change in fluorescence intensity after nerve stimulation in the group pretreated with phentolamine respectively even though the drug themselves had no effect on the fluorescence intensity in control animals.

It is concluded that if electrical stimulation of the splanchnic nerve can cause release of 5 HT from gut EC by an adrenergic mechanism which in good accordance with results in vitro (Peterson et al 1979) and electron microscopy finding (Newson et al 1979).

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#### D18

Comparison between the effects of diethyl-ethylamine on uterine, cardiac and skeletal muscles

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Diethyl-ethylamine (DES) one of the most potent uterine agents exerts marked effects on contractile activity of uterine, cardiac and skeletal muscles. DES in low concentrations has a strong relaxing effect on uterine smooth muscle (Batra & Bengtsson 1978) and it also exerts a negative inotropic effect on cardiac muscle. By contrast DES has a powerful potentiating effect on the twitch response of skeletal muscle (Rhen 1979). Studies on skeletal and cardiac muscles show that DES, in these low concentrations, does not affect the resting and action potentials. On the other hand DES has been shown to inhibit the calcium influx through the plasma membrane (Batra & Bengtsson 1978) as also the rate of resequestration of calcium by the isolated sarcoplasmic reticulum (Rhen 1978). Established data. The results support the idea that uterine and cardiac muscles predominantly depend on extracellular calcium influx for their contractile activity. An inhibitory effect of DES on the calcium influx is thus likely to be responsible for the decrease in contractile activity in these muscles. The contractile response of skeletal muscle is on the other hand not totally dependent on calcium influx from the exterior.

medium (Andersson & Edman 1974). In this muscle calcium is released from and resequestered by the sarcoplasmic reticulum (SR) forming a virtually closed cycle. The inhibitory effect by DES on the SR calcium pump may explain the potentiating effect on the twitch response of skeletal muscle fibres. This is supported by the finding that DES not only increases the twitch amplitude but also prolongs the relaxation phase in skeletal muscle. The relaxation of cardiac muscle is on the other hand not significantly affected by DES. This could be due to lower sensitivity of SR in cardiac muscle to DES or alternatively that liberation of calcium in cardiac muscle is largely accomplished by another system than SR.

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The metabolism of activator calcium in nonexcited myocardium evaluated from an analysis of frequency-interval relations

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The following model for the metabolism of activator calcium in the cardiac cell (Wohlfart 1979) has been used in order to explain the inotropic change that is produced by altering the stimulation interval. Calcium is released from a store Y into the myofibrillar space during the action potential. The mechanical response is thought to be directly related to the amount of calcium released. Relaxation occurs as calcium is withdrawn from the contractile system. A portion of the calcium (approximately 0.3 see below) is taken up by a store X and is added to the calcium that has entered the cell (and store X) during the action potential. The calcium contained in store X is finally transported to the releasable store Y. The complete replenishment of store Y after a release (at 37°C) requires a time period of 0.8 s. The amount of calcium in Y decreases slowly due to continuous leakage. The calcium inflow during the action potential is inversely related to the calcium contents of store Z. Release occurs from Z during the action potential and the replenishment is a time dependent process that occurs between action potentials. The rate of replenishment of Z is proportional to the amount of calcium in stores X and Y. The inflow of calcium during the action

potential will therefore be an inverse function of the inotropic state of the muscle.

The model explains why OCR optimum contractile response (Edman & Johansson 1976) is obtained. OCR is after a preceding stimulus. The increase in OCR after an extra stimulus (postextrasystolic potentiation) is due to: (1) a relatively small release of calcium from store Y by the extra stimulus and therefore small loss of calcium out of the cell and (2) an enhanced transport of calcium into the cell during the extra action potential (low content of calcium in Z). Postextrasystolic potentiation decays slowly if the muscle is left unstimulated due to the steady leakage of calcium from store Y. However, the potentiated state decays more rapidly if the muscle is stimulated as a relatively large fraction (approximately 0.7) of the released calcium leaves the cell. The model furthermore explains the biphasic rise in contractile force in response to increased stimulation frequency and also the enhancement of OCR that is seen after a period of high stimulation frequency (post-tetanic potentiation). The model predicts that OCR will increase with the stimulation frequency whereas the steady-state response will rise to a maximum value and then decline.

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## D 20

Gonadotropin interaction on ovine AIP formation in isolated theca cell from preovulatory rat follicles

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Preovulatory follicles were isolated from 2 days after treatment with a single injection of PMSG (Herlitz et al. 1976). From the follicles theca cells were mechanically isolated. The cells were subsequently incubated for 1 h. The presence or absence of LH or FSH and the accumulation of cyclic AIP in the assay plus medium was determined. In certain experiments the rats were injected with LH FSH or saline 2 h prior to sacrifice.

When the cells were isolated from rats killed before the endogenous gonadotropin surge FSH in vitro did not stimulate cyclic AIP formation. LH caused small but significant stimulation. When isolated in the evening, after the endogenous LH-FSH peak, the theca cells responded with marked increase in cAMP formation when exposed to LH in vitro whereas FSH in vitro had no effect.

When the cells were isolated in the morning, before the LH-FSH peak, and the rats were pre-treated with a single i.p. injection of FSH 2 h prior to sacrifice, the cells responded with marked increase in cyclic AIP formation.

subsequent in vitro exposure to LH. Thus pre-treatment with FSH which per se does not influence AIP formation made the cells more sensitive to LH.

These results indicate that the FSH component of the endogenous gonadotropin surge increases the sensitivity of the theca cell to LH. In vivo exposure to FSH seems to be essential for the development of responsiveness of the cyclic AIP system to LH in the preovulatory theca cell. As demonstrated earlier for isolated granulosa cells (Richards & J. S. 1975) FSH seems to be necessary for development of LH receptors on the theca cell.

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contraction dynamics; smooth muscle; t diff eqn  
 uers of act within.

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The mode of activation has been observed to influ-  
 ence the force-velocity relation in smooth muscle  
 (e.g. Murphy 1976) but systematic comparisons have  
 not been reported. We have determined series 1-  
 11 and force-velocity properties in individual  
 muscles under stimulation by AC current and  $K^+$ -high  
 medium, respectively. The experiments were performed on  
 preparations of two different kinds of smooth  
 muscle, the rabbit urinary bladder and the rat  
 portal vein. Each preparation was first stimulated  
 by repeated 2-5 s exposures to  $K^+$ -high solution  
 (100 mM  $K^+$  substituted for equal amount of  $Na^+$ )  
 then by AC-stimulation (50-60 Hz 2-3-5 V) each  
 lasting 3-10 s. The AC stimulation parameters were  
 adjusted to give the same isometric tension as the  
 $K^+$ -contraction. One isometric and k release was  
 made at the peak of each contraction with free-  
 load method in random tension records were  
 started in isometric state and subsequently analyzed  
 in computer (Hellstrand and Johansson 1979). The  
 amount of isometric release was calculated as was the  
 shortening velocity at 100 ms after release. The  
 force-velocity relation of the isometric element was  
 determined by fit to the equation

$$P = \exp(k \Delta L/L) B \quad (1)$$

The force-velocity data were analyzed according to  
 the Hill equation

$$V(P) = b(P_0 - P) \quad (2)$$

$P_0$  and  $V_{max}$  were determined as the intersections  
 of the force-velocity hyperbolic with the force and  
 velocity axes respectively.

The results are summarized in the table showing  
 $P_0$ ,  $k$  and  $V_{max}$  under the two stimulation condi-  
 tions. Mean  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$  by  
 Student's t-test for paired data.

		$P_0$ mmHg	$k$ [ct. eqn(1)]	$V_{max}$ length/s	
Bladder	AC	33	4	0.39	0.06
	$K^+$	33	4	0.26	0.03
Portal vein	AC	24	6	0.48	0.09
	$K^+$	21	4	0.34	0.06

Thus at 110 mmHg the stiffness of the isometric  
 element is the same for both stimulation conditions  
 whereas  $V_{max}$  is significantly lower in  $K^+$ -contrac-  
 tures both in bladder and portal vein.

The data give no information as to where in the  
 excitation-contraction sequence the observed  
 differences in  $V_{max}$  arises. It is possible that  
 different metabolic conditions exist or that struc-  
 tural changes in  $K^+$ -high medium as have been  
 reported by Jones et al. (1973) are of relevance.

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Extracellular pH and  $K^+$  concentration in  
 the cortex following t. ablates of  
ischemia

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 Anesthesiology University Hospital  
 Denmark

The clinical indication of t. ablates  
 isolates of cerebral ischemia is severe ly-  
 sis of the glucose peroxide t. ablates (Szkodowicz  
 & Hansen 1978). Because low pH was  
 suspected to be the cause of cellular damage  
 during ischemia, we measured both  
 extracellular pH and  $[K^+]$  in normoglycemic  
 and hyperglycemic rats. The potassium  
 measurement was included to provide an  
 index of the severity of ischemia.  
 Ischemia was induced by inflation of  
 a latex cuff around the neck and reduction  
 of MAP by removal of blood (Szkodowicz  
 & Hansen 1978). After craniotomy of  
 the parietal bone,  $[pH]$  and  $[K^+]$   
 were measured in the cortex by ion-selective  
 microelectrodes.

Plasma glucose was raised to the  
 level of ischemia by glucose administration  
 1 g/kg.

In normoglycemic rats plasma glucose  
 was 5.1  $\pm$  0.1 mM (SD). In hyperglycemic  
 rats 20 mM. Extracellular pH began to  
 fall immediately following the onset of  
 ischemia, decreasing from 7.15  $\pm$  0.05 to  
 6.55  $\pm$  0.20 in the normoglycemic rats and

from 7.10  $\pm$  0.06 to 6.12  $\pm$  0.06 in the hyper-  
 glycemic group. Extracellular  $[K^+]$  dis-  
 played the low and rapid phases of increase  
 in both groups.

The duration of the low phase averaged  
 1.6 min in the normoglycemic rats and 3.4  
 min in the hyperglycemic rats.

In both groups  $[pH]$  and  $[K^+]$  returned  
 to normal after ischemia. Normalization of  
 $[K^+]$  was complete after 3.5  $\pm$  0.5 min in the  
 normoglycemic rats and 4.1  $\pm$  0.6 min in the  
 hyperglycemic rats. Extracellular pH  
 was normal after 11.4 min in the normogly-  
 cemic and 20.6 min in the hyperglycemic  
 rats.

The results indicate that in the isoelec-  
 tric zone of hydrogen and potassium t. ablates  
 extracellular space of the cerebral cortex is  
 restored after 2 minutes of cerebral  
 ischemia despite marked differences in ex-  
 tracellular pH during ischemia and in the  
 time to impaired clinical indication.

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The cardiac output during rebreathing and steady state work. A comparison of two modifications of non-invasive technique

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A non-invasive method for measuring cardiac output ( $Q_r$ ) by determining the clearance of an inert soluble gas (acetylene) from closed rebreathing system by help of mass-spectrometry has recently been described (Sackner et al 1975 Triboase et al 1977). Earlier however many determinations of cardiac output in man by physiological methods were based on the acetylene method as described by Grollman 1929 ( $Q_{r1}$ ).

The aim of the present study was to compare the two methods during rest and during maximal exercise test.

8 human subjects were studied on two occasions. The results showed high reproducibility and an average difference of 10% in oxygen uptake ( $0.91 \pm 0.93$ ).  $Q_{r1}$  values were significantly higher than  $Q_{r2}$  values. The oxygen uptake was below 2.1 l/min. The difference was more pronounced during conditions where  $Q_{r1}$  values were almost 100% higher than  $Q_{r2}$  values. By including measurements of the oxygen uptake ( $VO_2$ ) and heart rate it was clearly shown that the central circulation is controlled during rebreathing leading to increased  $Q_{r1}$  values as well. This indicates the cardiac output during rebreathing while  $Q_{r2}$  records the cardiac output during steady state breathing. The rebreathing technique for determining  $Q_r$  by help of the  $VO_2$  (steady state)/

$VO_2$  (rebreathing) thus almost eliminates the difference between the two methods. The remaining differences can mainly be explained by methodological errors. As method 1 includes the pulmonary tidal volume which acetylene is solved in it. In this volume has been neglected method 2. The present study thus emphasizes that arterial output measured during rebreathing should be corrected to steady-state conditions in order to get proper results at rest and during low exercise level.

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D 24

Comparison of cat electroradiographic physiological recordings from cutaneous intramuscular and intradural level

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Examinations of the lumbosacral root potentials evoked by peripheral nerve stimulation have been performed in cats with cats (Happel 1975) and also with humans (Ertekin 1978).

In this report we compare conductivity values of lumbosacral roots by recording the potentials with electrodes placed on the skin surface intramuscularly and intradurally. We found that on the root level the seventh lumbar segment different recordings give significantly different conductivity values depending on the electrode locations.

The sciatic nerve of nine cats was stimulated both limbs separately separately with needle electrodes on the level of popliteal fossa. The responses were recorded with surface cup electrodes and intramuscular needle electrodes bipolarly. The electrodes were placed between the seventh lumbar and first sacral spine with the reference electrode between the sixth and seventh lumbar spine. After dissection of the paraspinal muscles intradural recordings were taken from the same level.

The latency was measured to the beginning of the first negative deflection and to the peak of the negative deflection (positivity downwards). The negative deflection of the potential was defined as the maximum amplitude of the response.

Means of measured values. Latency to the beginning of response with surface technique was 1.0 ms and to peak of negative deflection 1.1 ms. Maximum amplitude was 55 V.

Correspondingly in intramuscular recordings: 1.2 ms and 1.7 ms and 75 V. Correspondingly in intradural recordings: 1.3 ms and 1.8 ms and 350 V. The corresponding latency values to the beginning of response were significantly different between skin and intradural recordings (p < 0.01) and between skin and intramuscular recordings (p < 0.05).

We found significant difference in latency shown by various recording techniques at lumbosacral root level not only on the upper cord levels as Happel (1975) claims. It seems to be advisable to take account of difference in recording techniques when lumbar root conduction time are estimated.

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Distribution of ouabain binding sites  
in the dog nephron

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Sodium reabsorption in the thick ascend-  
 ing limb of Henle loop (TAL) seems to be  
 carried out by Na K ATPase which i present  
 in high concentr tion in the outer medull-  
 ary function f Na K ATY se in sodium reab-  
 sorption in the proximal tubules (PT) is  
 low lear and in terms f dry tissue  
 weight the acti ity f the enzyme i low in  
 PT compa ed to TAL and the distal tubules  
 (DT). However PT are long and tortuous  
 and the cell are large with high protein  
 content Hence the total amount of Na K  
 ATPase in PT might exceed the amount in  
 other nephron segment and the enzyme con-  
 centration in relation to tubul length  
 i f app oach that f TAL To examine this  
 hypothesis the rel tive concentration and  
 distribution f ouabain binding ites wer  
 estimated by quantitative autoradiography  
 (AQ) in dog kidneys

In ouabain 120 nmol/kg with specific  
 acti ity f 50 Ci/mol wa injected int  
 the renal artery f an anae thized dog  
 f this injection technique more than 80%  
 f the Na K ATPa activity i extinguished  
 and ouabain i exclusively bound t the  
 ATPase (Sej t ted 1979) in at ichio-

metri tio of i i Sections f r ARG we  
 obtained from biopsy treated with gl tar-  
 idehyde and OsO<sub>4</sub> dehydrated with ethanol  
 and embedded in Epon About 1% of the  
 activity was lost during prepar tion

The number of grains per p<sup>2</sup> of tubular  
 walls was identical in PT and DT but  
 higher by factor f 2.7 in TAL. Have  
 theless due to difference i tubular  
 wall thickness the number of grain pe  
 cross section did not diff in PT and TAL  
 but we more than 40% lower in DT Thus  
 the numbe of ouabain binding ites pe  
 tubular mm i identi l in PT and TAL  
 About 85% f the corti l grains were  
 loc li ed over PT Because 77% f all  
 renal ouabain binding ites are found in  
 the cortex (Sejersted 1979) PT contain  
 about 65% (0.83 ± 0.77 100%) f the total  
 renal amount of Na K ATPase and DT about  
 12%

Compared t transcell lar sodium sab-  
 sorption rates Na K ATPase seem to be  
 present in gre t excess in PT bec se as  
 much 60-70% of the absorbed sodium  
 passes by the paracellular route (Mathisen  
 et l 1976) In TAL and DT however  
 maximal sodium reabsorption i te could be  
 lose to the maximal pumping apa ity of  
 Na K-ATPase

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A telemetry study on deep body temperature of the Willow Tit (*Parus montanus*)

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Temperature measurements on Black capped Chickadee (*Parus atricapillus*) and the closely related Willow Tit (*Parus montanus*) by means of thermocouples have indicated that these birds when exposed to ambient temperatures below 0°C regulate their nocturnal body temperatures as far as 10-12°C below their normal diurnal temperature (Haftorn 1972 Chaplin 1974 Grossman & West 1977). The degree of temperature lowering differs significantly but this may be due to the different techniques used for body temperature measurements. The aim of the present study was to develop a method for continuous registration of core temperature of unrestrained small birds (10-12 g).

A small telemetry transmitter was developed for surgical implantation in the intraperitoneal cavity of the Willow Tit. The transmitter had a transmission range of 1 m and a sensitivity of 5% per °C. It was a nearly spherical disc with a diameter of 9 mm, a height of 4 mm and it weighed about 0.60 g. The signals from the transmitter were received through a commercial radio. The pulse rate depended on the temperature with increasing rate for increasing temperature. The transmitter

was calibrated in a saline bath over the range of temperature expected in the test animal (30-45°C). The transmitter was placed in the abdominal cavity under light ether anesthesia. As soon as the circadian behavioral cycle of the test bird was identical to that of the control birds, the experimental temperature measurements were performed. Immediately after completion of an experimental series, the transmitters were removed and retested to verify calibration.

The telemetric temperature recordings were compared to simultaneous thermocouple measurements. The circadian temperature cycle was correlated to the circadian behavioral cycle. The time and duration of deep sleep including the dynamics of thermoregulation when going to and waking from deep sleep were recorded.

The developed transmitters provided for remote recording of body temperature of unrestrained Willow Tits. The body temperature showed diurnal fluctuations and also transient fluctuations resulting from minor disturbance. Hence continuous temperature measurements are necessary if maximum degree of hypothermia shall be revealed.

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#### D 28

Behaviour patterns of cod released by electrical stimulation of the olfactory tract bundles

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The olfactory tract in cyprinid guppies and silurid is long and contains groups of myelinated and unmyelinated fibers (Doving & Gonne 1966 Doving 1967). The various portions of the tract terminate in different regions of the central nervous system indicating different functions (Finger 1975). When stimulating electrically the olfactory tracts in goldfish Grimm (1960) observed a feeding behaviour.

The caudal part of the olfactory tract of cod (*Gadus morhua* L.) can be dissected in four distinct bundles. Three of these bundles were cut symmetrically on both sides so that only one of the four bundles was intact. A pair of bipolar electrodes were hooked under the tracts connected to the skull and connected to a pulse stimulator. Weak electrical pulses in the order of 0.3 to 6.0 V and of 0.3 ms duration elicited in each a distinct and different behaviour: 1. the free swimming cod

Stimulation of the lateral part of the lateral olfactory tract made the fish tilt the head down to touch the bottom with the barbels and the first rays of the pelvic fins moving backwards. This behaviour seems adapted to food search for benthic prey.

Stimulation at low intensities of the medial part of the lateral tract induced a forward swim-

ming. Slightly higher stimulation intensities induced rapid snapping.

When the lateral part of the medial tract was stimulated the fish swam around with the snout above water. A quivering of the fish could be observed at high stimulation intensities.

Stimulation of the medial part of the medial olfactory tract made the fish lie quietly on the bottom and change skin coloration as if afraid.

These observations indicate the repertoire of behaviour patterns the olfactory system can mediate in the cod. The results support the notion of a special basis for olfactory discrimination and open new experimental means of investigating the neuroanatomical substrate for fish behaviour. The above observations will simplify the search for odorants that elicit these behaviour patterns.

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the importance of low molecular weight factor for human sperm motility

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Human seminal plasma normally has sperm motility promoting ability due to factors secreted by the human prostate (Lindholm 1974a). Androgen which is secreted by the prostate could be one factor of major importance in this respect (Lindholm & Eliasson 1974). In part due to its ability to protect the spermatozoa from "toxic" effects exerted by seminal plasma (Lindholm 1974b).

The aim of the present study was to further characterize seminal plasma factors promoting sperm motility and survival.

Seminal plasma from seven samples (30-5) with 0.2 living and motile spermatozoa and with progressive motility score (PMS) of 3-4 (i.e. good-excellent) were prepared by centrifugation, whereafter each supernatant was divided in two parts. One part served as control seminal plasma whereas the other part was dialyzed for 48h (4°C) against buffered saline, PSS (volume ratio 1:1000). The PSS was changed after 12h. The control was stored for the same time period (4°C). Thereafter one aliquot of washed spermatozoa obtained from pooled semen samples (quality shown) were incubated for three hours (37°C)

in each control and in each dialyzed seminal plasma specimen. Fructose and antibodies were added to all sperm suspension. Motility velocity and PMS were judged blind scored 0-12 and 3h.

The sperm liquefied incubated in the dialyzed seminal plasma had significantly lower percentage motile cells than those incubated in control seminal plasma (at  $p < 0.05$  3h  $p < 0.01$ ). The spermatozoa incubated in control seminal plasma retained progressive motility whereas those exposed to dialyzed seminal plasma totally lost this quality in four out of five cases. The sperm survival & living spermatozoa did not differ between the two groups.

These low molecular weight component (MW 10 000) are of significant importance for the sperm motility promoting ability of seminal plasma. Furthermore the decreased motility of the spermatozoa incubated in dialyzed seminal plasma is unlikely due to any toxic effects exerted by free ions. Ions removed by dialysis. Moreover, growth of androgen has no major role for the motility promoting ability of human seminal plasma. Since androgen is retained within the dialyzing sack.

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## D30

Interruption and glutamatergic control of hippocampal inhibitory interneurons and nucleus of the amygdala in the limbic system

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The septal complex is mainly responsible for fiber arising in the hippocampal formation (Swanson and Cowan 1977). Recent evidence suggests that one of these fibers may be glutamatergic as neurotransmitter. These glutamatergic fibers may control neurons in both septum and nucleus of the amygdala (Forsman and Wiles 1978). Maltre-Sprengsen et al. (1979). It was found that the septal complex identifies the nucleus of the septal complex. This may be achieved by means of local inhibition of the efferent glutamatergic neurons and which seems to do this by means of acetylcholine binding sites in the septum.

Local injection of kainic acid (2 µg) in the lateral septum and diagonal band was compared by decarboxylation of the glutamate decarboxylase (GAD) in the septum and decarboxylation of the choline acetyltransferase (ChAT) in the diagonal band. No change could be observed in the high affinity uptake of glutamate. Local injection in the lateral septum did not affect the present

neurotransmitter levels in the lateral septum hippocampus. However, 90% decrease in the ChAT activity in hippocampus could be detected after injection of kainic acid in the diagonal band only, whereas the GAD activity was unchanged. Simultaneously 20% decrease in ChAT was observed in the septum after this injection. All lateral limbic afferents from the septum and diagonal band are highly sensitive to glutamate septum and 50% reduction in the diagonal band. This reduction in the septum is consistent with the glutamate (10<sup>-5</sup> M) was able to release GABA in vitro. The GABAergic neurons are partially destroyed in the lateral septum by kainic acid whilst protected by flumazenil. The flumazenil protected by flumazenil afferents from hippocampus are mostly likely GABAergic neurons. This is supported by the release of GABA by glutamate in the septum by infusing the cholinergic neurons in the diagonal band. Control led by glutamate afferents from hippocampus providing the hippocampus with a very intense and back control of cholinergic fibers projecting from the diagonal band to hippocampus.

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Development of neurotransmitter parameters in lateral geniculate body visual cortex and colliculus superior of rats

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There is increasing evidence for glutamate as the transmitter in the corticofugal projections from visual cortex (VC) to lateral geniculate body (LGB) and colliculus superior (CS) (Lund Karlsson & Fonnum 1978)

It is well established that the high affinity (HA) uptake of glutamate in synaptosomes is a marker for glutaminergic nerve terminals (Fonnum et al 1979). We have therefore studied the postnatal development of these cortical projections in normal rats and in rats with unilateral visual cortex ablation at birth. HA GABA uptake and GAD activity were used as markers for GABAergic fibers and CHAT activity as a cholinergic marker.

In LGB and VC the HA glutamate uptake showed increasing activity from birth to adulthood. In contrast in SC the maximal uptake was higher at birth and reached a peak after 12 days of age. CHAT and GAD activities increased with age in all 3 regions. In all regions GABA uptake showed a 2-3 fold higher

activity at 12 days of age than in adult animals.

In some neonatal rats visual cortex was removed on one side. After 2 months the HA glutamate-uptake was similar contra and ipsilateral to the lesion in both SC and LGB. The uptake activity, however, was about 50% of unoperated adults. Such results may be explained from an aberrant crossing projection from the intact visual cortex (Mustari & Lund 1976). The ipsilateral decrease may reflect the loss of fibers crossing. These results describe the development of unilateral descendant glutaminergic fibers from visual cortex and that these after unilateral visual cortex ablation in the neonatal rat may also make contact on the contralateral side.

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### D 32

HDL cholesterol physical activity and diet in middle-aged men

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Previous studies have demonstrated an inverse correlation between high density lipoprotein (HDL) cholesterol and coronary artery disease (CAD) risk. Cross sectional studies have shown a higher HDL cholesterol concentration in physically active populations than in sedentary age-matched controls, the latter not as lean as the former.

In a preceding study (Kiens et al 1979) increased HDL-cholesterol concentrations occurred in healthy non-obese, initially sedentary middle-aged men after 12 weeks of physical conditioning. These findings occurred without changes in body weight or degree of adiposity. Dietary history data showed an increase in caloric intake without changes in the percentage distribution of fat, carbohydrate and protein, thus the absolute fat intake increased.

The aim with the present study was to investigate whether the observed increase in HDL-cholesterol was primarily due to the effect of physical activity or to the increased dietary fat intake. 23 healthy, regularly physically active (Vo<sub>2</sub> max 53.5 ± 3.9 ml/kg x min<sup>-1</sup>, mean ± SD) middle-aged men were studied. 12 were assigned to the experimental group and 11 to a control group. The experimental group were studied before and after 4 weeks of fat rich and after 4 weeks of carbohydrate-rich diet.

Preliminary results show no changes in HDL-cholesterol concentrations neither after fat rich diet compared to the normal before values (1.65 ± 0.12 vs 1.63 ± 0.09 mmol/l, N.S. mean ± SE) nor after carbohydrate rich diet compared to normal (1.59 ± 0.13 vs 1.63 ± 0.09 mmol/l, N.S. mean ± SE). No changes were seen in the controls. Fasting haemoglobin and body weight did not change during the study. This suggests that physical activity per se is the primary factor for the increase in HDL-cholesterol concentration.

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Theophylline induced release of renin from the renal kidney and its inhibition by adenosine

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It is well established that theophylline increases plasma renin activity in man (Witt et al. 1969 and others). One mechanism commonly assumed to be inhibition of AMP phosphodiesterase. In this communication we report that theophylline concentrations that have little or no effect on renal AMP phosphodiesterase increase renin release and that this effect is effectively antagonized by adenosine.

Renal kidneys were isolated and perfused continuously with Tyrode solution (Hedqvist et al. 1978). Renin release was determined by angiotensin I formed per hour and per perfused kidney. Radioimmunoassay kit for 1-angiotensin I radioimmunoassay kit.

Theophylline caused dose-dependent increase in renin release from the kidney. A significant effect was obtained with theophylline 1 mM and with 10 mM renin release was more than doubled. In these concentrations theophylline has virtually no effect on AMP phosphodiesterase activity in the renal kidney (Hedqvist et al. 1978). Adenosine inhibited spontaneous release of renin, and counteracted the enhancing effect of theophylline. The inhibitory effect of adenosine on renin release by theophylline was dose-dependent and was also counteracted by raising the theophylline concentration, suggesting competitive antagonism.

It was shown (Fredholm & Hedqvist 1978) that

renal nerve stimulation can induce renin release which correlated well to the induced vasoconstriction. In the present study renal nerve stimulation led to an increased release of renin which could be blocked by 1  $\mu$ M adenosine. Renin release was also depressed when the vasoconstrictor response to nerve stimulation was enhanced by reducing the perfusion medium by 10%. On the other hand, renal release of renin was enhanced after adrenoceptor blockade which abolished the vasoconstrictor response and the release of renin (Fredholm & Hedqvist 1978).

Our results indicate that adenosine inhibits renin release as well as that induced by theophylline or nerve stimulation. Since adenosine acts through its concentration which is of the same order of magnitude that found to be released from the kidney it is possible that the enhancing effect of theophylline on renin release is at least in part an late action with endogenous adenosine. The results are also consistent with the opinion that adenosine may play a role in the regulation of renin release.

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#### D 34

Isolation and characterization of small polypeptide hormones and chromaffin granules from the bovine adrenal medulla

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Of the six known proteins in components of the bovine chromaffin granule membrane (Viallet 1976) three have been identified in large amounts:  $\alpha$ -melanocyte stimulating hormone (MSH),  $\beta$ -melanocyte stimulating hormone (BMSH) and chromogranin B (CG-B) (Lagerström 1976). In the bovine adrenal medulla MSH and chromogranin A have been found.

In this study we have compared the protein patterns of small dense cores isolated from previously known with those of chromaffin granules of the rat.

Rat chromaffin granules were isolated according to Smith & Viallet (1967) and small dense cores isolated according to Fried et al. (1978). The subcellular fractions were subjected to SDS-polyacrylamide gel electrophoresis in 3.5% gel (Uver & Osborn 1969). The ability of the bands relative to the tracking dye was calculated and the apparent molecular weight was estimated using standard plots with proteins of known molecular weight.

The rat chromaffin granule membranes contained no major proteins with similar apparent molecular weight to bovine MSH, with dimeric 105 000 dalton and monomeric 107 000 dalton. The other major membrane proteins were found at 10 000 and 36 000 dalton. A band at 36 000 dalton indicated possible presence of chromogranin.

Bovine MSH, the soluble contents contained two major proteins, 100 000 and 89 000 dalton, in contrast to bovine chromaffin granules where there is one major protein in chromogranin A at 80 000 dalton.

The small dense cores of adrenal fraction also contained protein with similar mobility to bovine MSH but this was not a major protein. A strikingly large component was found at 28 500 dalton, the mobility of chromogranin B. A major protein line with mobility similar to chromogranin was present.

The small dense cores of adrenal fraction previously by biochemical method been shown to contain MSH and  $\beta$ -melanocyte stimulating hormone (Fried et al. 1978). These results indicate that also chromogranin B is present that MSH is not a major protein and that chromogranins are very minor components if present at all. Regarding chromogranins there seems to be differences between rat and bovine chromaffin granules.

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Respiratory functions in highly trained - and normal boys during puberty

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The aim of the present study was to evaluate the qualitative changes in the following respiratory functions: Vital capacity (VC), maximal pulmonary ventilation ( $V_p$ ), maximal tidal volume ( $V_T$ ) and maximal respiratory frequency ( $f$ ).

These parameters were measured in a group of six highly trained boys (group 1) and in a group of 5 normal boys (group 2) from 1.5 years before till 1.5 years after peak height growth velocity (PHV) (for further details see Andersen and Froberg 1979).

VC measured in supine position was found to be 10% lower in group one than in group two before PHV. From PHV occurred until 1.5 years after the two groups were similar. Corrected for differences in height ( $VC \cdot a \cdot h^{-1}$ ) similarity was found until one year before PHV. Then VC increased relatively more in group one resulting in a 10% higher VC in this group from 0.5 years after PHV. Maximal  $V_p$  in group one increased linearly from  $85 \text{ l} \cdot \text{min}^{-1}$  1.5 years before PHV to  $135 \text{ l} \cdot \text{min}^{-1}$  1.5 years after. The corresponding values in group two were  $75 \text{ l} \cdot \text{min}^{-1}$  and  $115 \text{ l} \cdot \text{min}^{-1}$ . This increase was not linear. The difference in  $V_p$  between the two groups was not related to differences in maximal  $V_T$  but alone to differences in  $f$ . The latter was 66.5 in group one 1.5 years before PHV and decreasing to 60.3 1.5 years after PHV. In group two they were 55.7 and 49.5 respectively. The ventilatory equivalent was close

to 38 liter per liter oxygen in both groups during the period.

The results indicate that training during puberty induces qualitative changes in VC and in  $f$ . This could partly explain the higher maximal oxygen uptake found in the highly trained group.

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ANDERSEN B & FROBERG 1979 Acta Physiol Scand this issue

## D 36

Circulatory parameters and muscular strength in trained and normal boys during puberty

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The present paper presents circulatory parameters and muscular strength measurements in a group consisting of six highly trained boys (group one) training more than 7.5 hours per week and in a group consisting of 5 normal boys (group two) training less than 3.5 hours per week over a period of 5 years starting at the age of eleven years. The obtained measurements were related to the time of peak height growth velocity (PHV). Comparisons between the two groups are made from 1.5 years before PHV till 1.5 years after. (For further details on  $VO_2$  and respiratory functions see Andersen and Froberg).

The hematocrit (Hct) values were close to 40% during the whole period and was the same in the two groups while hemoglobin concentration (Hb) increased from 13 to 14.9 gms per 100 ml in the same period. The increase was similar in the two groups except during PHV and 0.5 years before. Here the Hb concentration was constant in group one while it increased constantly in group two. In group one the concentration was 95% of the values found in group two. The average maximal HR was 198 in group one and five beats higher compared to group two. If corrected for height the difference was 1.2%. Net efficiency was found to increase from 21.5% to 22.5% at the submaximal work loads over the years investigated with no systematic differences between the groups. Maximal isometric strength was measured in a strain

gauge apparatus. The strength in the back muscles was found to increase from 460 N to 740 N in group one. This was 30% higher than found in group two. From 1.5 year before PHV to 0.5 years after PHV the difference between the groups was only 10% if corrected for differences in height ( $F \cdot a \cdot h^{-2}$ ). The same pattern was found for the elbow flexors.

The study indicates that when the pubertal growth spurt takes place highly trained boys have less advantage of the training of muscle strength compared with the period before and after PHV. This is in accordance with results obtained in a cross-sectional study (Lamert et al). The circulatory changes are in accordance with the literature except for the 5% lower Hb concentration values in group one. It may reflect an adaptation of blood viscosity to training.

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maximal oxygen uptake and lactate concentration in highly trained and normal boys during puberty.

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Results obtained in literature are contradictory concerning the effect of training on boys during puberty (Bablon 1979, Ekoye et al 1978, >ber Kristiansen & Klisouras 1976). One reason could be that results generally are related to chronological age, not to development. The present longitudinal study deals with 11 boys out of a group of 29, investigated over a period of 5 years starting at the age of 11 years. Six of the boys (group 1) trained more than 7.5 hours per week. Most of the training partly supervised by this institute was performed in soccer club. These boys were compared with five boys (group 2) who trained less than 3.5 hours per week.

Height, weight, oxygen uptake, pulmonary ventilation, heart rate and blood lactate concentration were measured every half year.

The tests were performed on a bicycle ergometer at two submaximal and two supermaximal work loads. The changes in maximal oxygen uptake and lactate concentration were related to that half of a year where the increase in height was the highest (Peak Height Growth Velocity, PHV). It was possible to compare the results from all the boys in the two groups from 1.5 years before PHV until 1.5 years after.

It was found that maximal oxygen uptake in group one increased from  $2.2 \pm 1.0 \text{ l O}_2 \cdot \text{min}^{-1}$  to  $3.5 \pm 1.0 \text{ l O}_2 \cdot \text{min}^{-1}$  in the period constantly being  $250 \text{ ml O}_2 \cdot \text{min}^{-1}$  higher than the values obtained in group two. Concerning blood lactate concentration, the maximal values were found to be  $10 \text{ mmol l}^{-1}$  in group one over the period. In group two the values were  $7.5 \text{ mmol l}^{-1}$  1.5 years before PHV increasing till  $9 \text{ mmol l}^{-1}$  5 years before PHV and being so the rest of the period.

If sensitivity to training is defined as change in the trained parameter per unit of time the two groups were equally sensitive concerning maximal oxygen uptake regardless of the greater amount of training performed by group one. In regard to maximal blood lactate concentration, this was only so from 5 years before PHV. This suggests that an external stimulus such as training apparently can not override the influence of growth during this period. This may be discouraging, but could be outweighed by the qualitative changes described in the following papers.

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#### D 38

The relationship between isometric strength in boys and girls and developmental parameters.

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Muscle strength is related to several parameters: muscle fiber types, degree of training, sex and age. Before puberty there is hardly no difference between the two sexes and only slight sexual increase in strength when puberty occurs. Boys start to increase muscle strength rapidly while this is not seen in girls. During this period chronological age is a poor indicator of development. The present study investigated the relationship between maximal isometric strength and to the following parameters: chronological age, height, weight, skeletal age, dental age, Tanner's adolescent stages (Tanner 1962) and predicted onset of pubertal growth spurt. These parameters were measured in randomly selected group of 224 children chronological aged 9 to 17 years. Each age group had an equal number of boys and girls. The strength in the arm flexors (100% MVC) was measured in strain gauge apparatus. Skeletal age (Greulich & Pyle 1959), dental age (Demirjian et al 1973) and predicted onset of normal growth spurt (Hs) (Heim et al 1973) were obtained from radiograph of the hand and of the wrist. The results show that for the boys maximal isometric strength increased up to 12 years of age and again at 15 years of chronological age. A similar pattern was also found when isometric strength was related to height, weight and predicted onset of pubertal growth spurt. Here the lack

of increase in muscle strength was seen from 154 cm to 162 cm, 42 kg to 47 kg and 1.5 years before Hs to Hs respectively. Compared to skeletal age it increased up to 12 years with an increase like the one seen in the girls and then with steeper increase. The girls always had somewhat lower muscle strength than the boys. The relationship for the girls showed constant increase up to 2 years after Hs when isometric strength became constant when related to chronological age, weight, height or skeletal age. The strength increased constantly within the observed intervals.

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The relationship between isometric strength in girls and boys and the serum concentration of FSH

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Muscle strength is related to several parameters such as the degree of training, age and sex. Strength increases slightly until the age where puberty occurs, then it is well known that muscle strength increases rapidly in boys. It is also known that during this period changes occur in the serum concentration of several hormones. The present study investigated the relationship between isometric muscle strength and the serum concentration of FSH (follicle stimulating hormone). Measurements of maximal isometric muscle strength in the arm flexors at 90° elbow flexion and blood samples were obtained twice interspersed by 5 weeks. A randomly selected group of 91 girls and 118 boys aged 9 to 17 years were used. No systematic change in strength or FSH was found between the measurements. FSH was measured using a radioimmunoassay method. The relationship between force and FSH can be expressed for the girls as force =  $0.058 \times \text{FSH concentration} + 9.6405$  with a correlation coefficient of  $r=0.443$  and  $n=182$ . For the boys it was force =  $0.3544 \times \text{FSH concentration} + 10.2118$  and  $r=0.6423$  and  $n=236$ . The relationship was found to be linear for both boys and girls. The values of FSH for boys were between 2.2 and 20 m IU/ml and the force between 60 and 250 N. For the girls the values observed were between 3.3 and 29 m IU/ml and the force between 59 N and 185 N. The linear relationship found for the

boys are not in accordance with results obtained when force is related to chronological age, onset of pubertal growth spurt or skeletal age (Larrent et al 1979). For the boys the statistically explained variation was 40% while it only was 10% for the girls. In both cases the relationship was highly significant,  $t_{p12.88}$  and  $t_{p6.84}$  and  $P < 0.001$ .

In conclusion the investigated relationship reflects poorly the changes in muscle strength in this cross sectional group. One of the reasons could be that increase in serum level of FSH is reported to start in the prepubertal period (Ferrin et al 1974).

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## D 40

Release of endogenous noradrenaline from blood perfused rabbit kidney. Influence of prostaglandin  $E_2$  and indomethacin

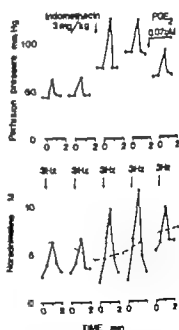
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The hypothesis that prostaglandins of the  $E$  type ( $PGE_2$ ) play a significant modulatory role in adrenergic neuroeffector transmission (cf Hedqvist 1977, Ann Rev Pharmacol Toxicol 17:259-279) was tested in blood perfused rabbit kidneys *in situ*. Kidneys were perfused at constant rate (10 ml/min) and the renal nerves were intermittently stimulated (3 Hz, 1 msec for 30 sec). Noradrenaline (NA) in renal arterial and venous blood was determined by high performance liquid chromatography and electrochemical detection.

Renal nerve stimulation caused vasoconstriction and increased NA in the venous effluent by  $7 \pm 1$  nM ( $n=22$ ). Indomethacin (3 mg/kg i.v.) increased renal perfusion pressure and enhanced both the stimulation-induced outflow of NA and the vasoconstrictor response (Fig.). NA outflow was increased by  $87 \pm 30\%$  ( $n=5$ ,  $p=0.05$ ).  $PGE_2$  (0.07  $\mu$ M) reduced stimulation-induced NA outflow to  $51 \pm 9\%$  ( $n=5$ ) of control values and attenuated the vasoconstrictor response. The effects of  $PGE_2$  were similar after treatment with indomethacin.

The present results obtained *in situ* determination of endogenous NA in the blood perfused kidney *in situ* provide considerable support for the view that  $PGE_2$  may be a significant feedback modulator of transmitter secretion in adrenergically innervated tissues.



Effects of indomethacin and  $PGE_2$  on responses to renal nerve stimulation

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# permeability of the peritubular capillary membrane in the renal kidney

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The fluid reabsorption across the peritubular capillary membrane will be governed by the Starling forces operative across the membrane and its permeability. The permeability of the membrane was estimated from its hydraulic conductivity and its permeability to 125-I-paraaminohippuric acid (PAH) to inulin, albumin and gammaglobulin.

Hydraulic conductivity was investigated from the driving forces and fluid transport. Hydrostatic intracapillary and interstitial (a.g. osmotic) pressure was measured with direct punctures. In venous colloid osmotic pressure was determined from the protein concentration in renal hilar lymph. Osmotic pressure along the peritubular capillary was calculated from the protein concentration in afferent arterial blood and in renal venous blood. The fluid transfer was determined from single nephron filtration and the inulin concentration in distal tubular fluid.

Using osmotic concentration as the driving force was 1.1 mm Hg and the amount of fluid reabsorbed 11.5 nl/(min 100 g wet) i.e. hydraulic conductivity of 0.96 nl/(min 100 g mm Hg).

This would mean total pore area over pore length 1/10 of about 2 cm<sup>2</sup>/100 g at as calculated from

Poiseuille's law for radii between 3 and 15 nm.

From microinjections of PAH into the peritubular capillary blood the flux across the membrane could be calculated and this resulted in  $A_p/dx$  of about 0.3 cm and was also found to be very insensitive to the pore radius. The transport of the proteins was derived from the concentration in systemic plasma and hilar lymph. Albumin and gammaglobulin concentrations was determined separately from their concentration. The permeability  $A_p/dx$  was here estimated 2 and 3 cm respectively for 10 nm pores.

Combining all data the total pore area of 1 pore length of 0.1 cm was estimated to  $2 \cdot 10^{-5}$  cm<sup>2</sup> corresponding to about 1% of the total capillary area. The effective radius of the pores for reabsorption will range from 3 to 7 nm.

During extracellular volume expansion the driving forces decreased but the permeability increased in proportion resulting in an unchanged absolute reabsorption.

It is concluded that the permeability of the peritubular capillary membrane is very high compared to that of other capillary beds but very similar to that for the glomerular capillary membrane.

## D 42

Impulse for both Na and Ca<sup>2+</sup> dependent  
impulse 1 log current in Bell's potential  
impulse

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Key of the neurons of the lateral pons  
display particular so-called impulse  
dependent firing patterns (Colding-  
Jørgensen 1975) because each firing  
interval depends on the presence and the  
size of previous impulses.

This behaviour is due to accumulation  
of hyperpolarizing current which is  
triggered by the depolarization during  
the impulse and which decays exponentially  
between the impulses. The reversal  
potential of the current is -40 to -70  
mV which strongly suggests that the  
current is carried by K<sup>+</sup> ions (Colding-  
Jørgensen 1977).

Further analysis has revealed that the  
current changes in Ca<sup>2+</sup> free medium  
and that Ca<sup>2+</sup> reduces the current to less  
than 10% without changing the reversal  
potential. The first observation implies  
that the current depends on the extracellular  
Ca<sup>2+</sup> concentration. The second  
fact implies that Ca<sup>2+</sup> plays an important  
role in the current if reaction. However,  
the Ca<sup>2+</sup> does not participate in the  
current itself.

Replacing Na<sup>+</sup> with Tris<sup>+</sup> results in a  
reduction of the current to 40-50% of  
its normal value but also in hyperpolarization  
of the reversal potential of  
5-15 mV. This implies that extracellular  
Na<sup>+</sup> is necessary for the full current but  
also that Na<sup>+</sup> participates in the current  
itself. Replacing Cl<sup>-</sup> with isethionate  
does not give any significant change in  
the current.

The decline of the hyperpolarizing current  
after depolarization of 10 mV is  
in all cases mono-exponential so that  
current is most probably passing through  
homogeneous population of pathways.

It can therefore be concluded that the  
current appears to be carried through the  
membrane by a mechanism which is dependent  
on the presence of both Na<sup>+</sup> and Ca<sup>2+</sup>  
in the bathing medium and that the current  
mainly is carried by K<sup>+</sup> ions but to  
small extent also by Na<sup>+</sup> ions. Whether  
the controlling ions act on the outside  
or the inside of the membrane is not yet  
determined. Preliminary experiments show  
that the intracellular Ca<sup>2+</sup> concentration  
increases during the depolarization and  
decreases the current decreases but  
the effect of Na<sup>+</sup> is still unknown.

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The relationship between isometric strength in girls and boys and the serum concentration of FSH

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Muscle strength is related to several parameters such as the degree of training age and sex. Strength increases slightly until the age where puberty occur then it is well known that muscle strength increases rapidly in boys. It is also known that during this period changes occur in the serum concentration of several hormones. The present study investigated the relationship between isometric muscle strength and the serum concentration of FSH (follicle stimulating hormone). Measurements of maximal isometric muscle strength in the arm flexors at 90° elbow flexion and blood samples were obtained twice interspersed by 5 weeks. A randomly selected group of 91 girls and 118 boys aged 9 to 17 years were used. No systematic change in strength or FSH was found between the measurements. FSH was measured using a radioimmunoassay method. The relationship between force and FSH can be expressed for the girls as force

$0.058 \times \text{FSH concentration} + 9.6405$  with a correlation coefficient of  $r=0.443$  and  $n=182$ . For the boys it was force  $0.3544 \times \text{FSH concentration} + 10.2118$  and  $r=0.5423$  and  $n=236$ . The relationship was found to be linear for both boys and girls. The values of FSH for boys were between 2.2 and 20 m IU/ml and the force between 60 and 250 N. For the girls the values observed were between 3.3 and 29 m IU/ml and the force between 59 N and 285 N. The linear relationship found for the

boys are not in accordance with results obtained when force is related to chronological age, onset of pubertal growth spurt or skeletal age (Lambert et al 1979). For the boys the statistically explained variation was 40% while it only was 20% for the girls. In both cases the relationship was highly significant  $t_{0.1288}$  and  $t_{0.64}$  and  $P < 0.001$ .

In conclusion the investigated relationship reflects poorly the changes in muscle strength in this cross sectional group. One of the reasons could be that increase in serum level of FSH is reported to start in the prepubertal period (Ferin et al 1974).

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#### D 40

Release of endogenous noradrenaline from blood perfused rabbit kidney: influence of prostaglandin  $E_2$  and indomethacin

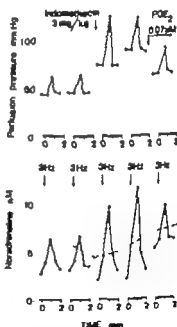
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The hypothesis that prostaglandins of the E type (PGE) play a significant modulatory role in adrenergic neuroeffector transmission (cf Hedqvist 1977 *Ann Rev Pharmacol Toxicol* 17:259-279) was tested in blood perfused rabbit kidneys *in situ*. Kidneys were perfused at constant rate (10 ml/min) and the renal nerves were intermittently stimulated (3 Hz, 1 msec for 30 sec). Noradrenaline (NA) in renal arterial and venous blood was determined by high performance liquid chromatography and electrochemical detection.

Renal nerve stimulation caused vasoconstriction, and increased NA in the venous effluent by  $7.8 \pm 1.1$  nM (n 22). Indomethacin (3 mg/kg i.v.) increased renal perfusion pressure and enhanced both the stimulation-induced outflow of NA and the vasoconstrictor response (Fig.). NA outflow was increased by  $87 \pm 30\%$  (n 5,  $P < 0.05$ ). PGE<sub>2</sub> (0.07 μM) reduced stimulation induced NA outflow to  $51 \pm 9\%$  (n 5) of control values and attenuated the vasoconstrictor response. The effects of PGE<sub>2</sub> were similar after treatment with indomethacin.

The present results obtained with determination of endogenous NA in the blood perfused kidney *in situ* provide considerable support for the view that PGE<sub>2</sub> may be a significant feedback modulator of transmitter secretion in adrenergically innervated tissues.



Effects of indomethacin and PGE<sub>2</sub> on responses in renal nerve stimulation

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permeability of the peritubular capillary membrane  
in the rat kidney

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The fluid reabsorption across the peritubular capillary membrane will be governed by the Starling forces operative across the membrane and its permeability. The permeability of the membrane was measured from its hydraulic conductivity and its permeability to (2S-l)-paraminohyppuric acid (PAN) 3- $\alpha$ -amino, albumin and gammaglobulin.

Hydraulic conductivity was investigated from the driving forces and fluid reabsorption. Hydraulic conductivity and interstitial pressure was measured with direct punctures. Interstitial fluid velocity was determined from the protein concentration in renal hilar lymph. Oncotic pressure along the peritubular capillary was calculated from the protein concentration in the renal arterial blood and in renal venous blood. The fluid transfer was determined from single nephron filtration and the sodium concentration in distal tubular fluid.

During antidiuretic conditions the mean net driving force was 34 mm Hg and the amount of fluid reabsorbed 11.5 ml/min (100 g wt) and hydraulic conductivity of 0.94 ml/min (100 g wt mm Hg).

This would mean total pore area over pore length,  $A_p/l$  of about 2 cm<sup>2</sup>/100 g wt as calculated from

Poiseuille law for radii between 3 and 15 nm

From microinjection of PAN into the peritubular capillary blood the flux across the membrane could be calculated and his resulted in  $A_p/l$  of about 0.5 cm<sup>2</sup> and was too low to be very sensitive to the pore radius. The transport of the protein was derived from the concentration in systemic plasma and hilar lymph. Albumin and gammaglobulin concentrations was determined separately from their concentration. The permeability  $A_p/l$  was here estimated at 2 and 3 cm<sup>2</sup> respectively for 10 nm pores.

Combining all data the total pore area for pore length of 0.1 cm was estimated to  $2 \cdot 10^{-3}$  cm<sup>2</sup> corresponding to about 1% of the total capillary area. The effective radius of the pores for each nephron will range from 3 to 7 nm.

During extracellular volume expansion the driving force decreased but the permeability increased in proportion resulting in an unchanged absolute reabsorption.

It is concluded that the permeability of the peritubular capillary membrane is very high compared to that of other capillary beds but very similar to that for the glomerular capillary membrane.

## D42

Evidence for both Na and Ca<sup>2+</sup> dependent hyperpolarizing current in *Helix pomatia*

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Many of the processes of *Helix pomatia* display particular so-called impulse dependent firing patterns (Colling-Jørgensen 1975) because each firing interval depends on the presence and timing of previous impulses.

This behaviour is due to accumulation of hyperpolarizing current which is triggered by the depolarization during the impulse and which decays exponentially after the impulse. The reversal potential for the current is -60 to -70 mV which strongly suggest that the current is carried by K<sup>+</sup> ions (Colling-Jørgensen 1977).

Further analysis has revealed that the current vanishes in Ca<sup>2+</sup> free medium and that Co<sup>++</sup> reduces the current to less than 10% without changing the reversal potential. The first observation implies that the current depends on the extracellular Ca<sup>2+</sup> concentration the second that influx of Ca<sup>2+</sup> plays an important role in the current formation. However the Ca<sup>2+</sup> does not participate in the current itself.

Replacing Na with Tris results in reduction of the current to 40-50% of its normal value but also in hyperpolarization of the reversal potential of -5 to -15 mV. This implies that extracellular Na is necessary for the full current but also that Na participates in the current itself. Replacing Cl with isethionate does not give any significant change in the current.

The decline of the hyperpolarizing current after depolarization of 50 mV is in all cases mono-exponential so the current is most probably passing through homogeneous population of pathways.

It can therefore be concluded that the current appears to be carried through the same site by a mechanism which is depending on the presence of both Na and Ca in the bathing medium and that the current mainly is carried by K<sup>+</sup> ions but to small extend also by Na ions. Whether the controlling ions act on the outside or the inside of the membrane is not yet determined.

Preliminary experiments show that the intracellular Ca<sup>2+</sup> concentration increases during the depolarization and decreases as the current decreases but the effect of Na is still unknown.

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Local cerebral glucose consumption during  
a single cortical spreading depression

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Laco Co tic l sp ding D pr io (CSD)  
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 r b l o t t io i flu c Th  
 CSD i mp id by l c l ch g of DC  
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 f K+ Na+ Cl d Ca++ d m v t  
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 CSD f r 45 min (Shinohar a al 1979)  
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 o i w f i it d i g th p  
 g of i gl CSD 10 ft i t v  
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 d D gl di l 2 = l li  
 v o t f th t Th [K] w = i  
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[illegible]

rCBF	44±2	ml/100g/min
Tmax	157±11	μmol/100g/min
rCBG	29±5	μmol/100g/min

h i p b i p r t f t h i g h t h i  
 p h h w i t h f i g i h c h  
 p t i l v t a w t d t t p l  
 v t b y t h k o w f p g r i f  
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 S I M M O R A s a 1 1 9 7 9 S c i e n c e 2 0 3 1 2 8 - 1 9 0  
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## D 44

The basement membrane thickness and mitochondrial content of brain capillaries in normal and postischemic human rats

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The volume of mitochondria in brain capillary endothelial cells exceeds the content in capillary endothelial cells from a number of other rat tissues and an excess metabolic work capability of the blood brain barrier has been suggested (Oldendorf et al). In order to define the structural relationship of the blood brain barrier to work capacity and diffusing capacity in hepatic encephalopathy where changes in the barrier may be an etiologic factor the mitochondrial content of the endothelial cells and the harmonic mean thickness of the basement membrane of the brain capillaries were measured in sham operated and portocaval shunted rats.

The mitochondrial content was measured by automatic image analysis (Laursen & Dierker) and the harmonic mean thickness of the brain capillary basement membrane was determined from orthogonal intercepts measured on a reciprocal logarithmic scale as proposed by Jansen et al. The mitochondrial content in brain capillary endothelial cells was 5.97 (3.25 to 8.75) per cent after sham operation, 7.64 (3.57 to 70)

per cent after 10 days PCA 5 78  
(3 55-8 51) per cent after 30 days PCA  
and 5 73 (2 60-10 01) per cent after 10  
weeks PCA (median with 25 75 percentiles).  
The harmonic mean thickness of the m  
pill ry basement membrane wa 57 8 nm  
after sham operation #4 6 nm after 10  
days PCA 63 2 nm after 30 days PCA and  
60 0 nm after 10 weeks PCA The slightly  
incre d val e of the basement mem  
thickness after 30 days PCA was the only  
diff rance betwe all the groupe

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# Control of muscle properties by pattern of impulse activity

by

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Cross reinnervation experiments have established that neural influences largely determine the differentiation of mammalian skeletal muscle into fast and slow types (Buller Eccles & Eccles 1960). However, experiments with innervated muscles do not distinguish between transformations in the muscle caused by changes in muscle activity per se or by changes in flow of trophic substances resulting from altered nerve activity. Earlier experiments on denervated muscle have shown that transformations occur with different patterns of stimulation in the absence of neural influences (Lomo Westgaard & Dahl 1974). In the present experiments this result is confirmed and extended by showing that denervated muscle may obtain very different contraction speed, strength and endurance dependent on the particular pattern of activity imposed.

Stimulating electrodes from external stimuli (platinum plates or teflon coated steel wire) were implanted around the denervated soleus muscle of male Wistar rats. Stimulation was maintained for 3 to 15 weeks. In the acute experiment measurements were made at optimal length and at a temperature of 35-36°C. The soleus was either freed extensively (Lomo & al 1974) or subjected

to minimal dissection of the distal end to preserve blood supply and obtain a reliable measure of endurance.

Four patterns of stimulation were used, namely brief 100 Hz trains (0.67 sec) repeated either frequently (every 67 sec) or infrequently (every 1 h 57 min 50 sec) giving mean frequencies of 1 and 0.01 Hz; and 10 Hz trains (9 sec every 10 sec or 1 sec every 70 sec) mean frequency 9 and 1 Hz. Muscles stimulated with trains of 100 Hz became fast while those stimulated with trains of 10 Hz remained slow irrespective of mean frequency. The change in contraction speed resulted in the steep part of the tension vs. frequency curve (where muscle tension is most sensitive to small changes in rate of stimulation) moving towards the imposed frequency. Muscles receiving frequent stimulation became stronger and more fatigue resistant than those stimulated less at the same frequency. Muscles stimulated at 100 Hz produced larger tetanic force than muscles receiving much more frequent stimulation at 10 Hz.

These results show that the muscle is able to adjust a number of intrinsic properties according to the external demand on it. Frequency and amount of stimulation are important factors in determining the new intrinsic muscle properties.

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## D 48

The role of adrenergic and pancreatic hormonal secretion and muscular glycogenolysis in running in the rat  
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During prolonged exercise insulin secretion decreases and glucagon secretion increases. We have reported that swimming in the rat causes changes in the hormonal control of the adrenal medulla (DM). Compared to control DM, rats also have higher muscular glycogen concentration and diminished glycogenolysis during swimming (Richter & al 1979). These effects of DM in swimming may be secondary to changes in swimming intensity or efficiency.

We have now studied if similar effects of DM can be demonstrated in running and if they are reversed by exogenous adrenaline. 24 rats were DM and 16 sham-operated (C) rats. The rats were anesthetized and implanted with catheters in the aorta and the vena cava. They were implanted 2 weeks later with the catheters tested (16) for 45 min or an 1 h metabolic chamber (24) (Richter & al 1979). Running DM- rats had saline (DM-S) or adrenaline (DM-A) (to the same plasma concentration as in C-rats) infused blood samples were drawn before and during exercise immediately after the 45 min metabolic chamber samples we take the 45 min metabolic chamber samples we take

$\dot{V}_{O_2}$  was identical in C and DM- rats increasing from 2.4 ± 0.1 (mean ± SE) l/min to 4.9 ± 0.1 ml O<sub>2</sub> (STPD) (min 100 g) during exercise. C rats heart rate (HR) increased from 394 ± 14 to 469 ± 13 beats/min during exercise. In running DM-A rats only increased significantly in DM-A rats (from 366 ± 13 to 445 ± 15) l/min and DM-A rats blood glucose increased from 6.0 ± 0.1 mM to 8.2 ± 0.5 and 7.0 ± 0.3 respectively whereas it decreased

to 5.6 ± 0.3 mM in DM-S rats during exercise. Plasma insulin concentration was the same in resting and running rats. In C rats the plasma glucose concentration increased from 11.2 ± 3.4 mM during exercise and in DM-A and DM-S rats from 13.2 ± 2.9 and 28.2 ± 2.2 respectively. By plotting these hormonal changes relative to the changes in blood glucose it appears that compared to DM-S rats insulin concentration was depressed and glucagon concentration increased in C and DM-A rats during running. C rats muscular glycogen decreased from 17.1 ± 9.2 mmol/kg during exercise and in DM-S and DM-A rats from 27.2 ± 19.2 and 10.1 respectively. The decrease being larger in DM-A than in DM-S rats. C rats blood lactate increased from 1.36 ± 0.10 to 2.37 ± 0.46 mM during exercise and in DM-S and DM-A rats from 1.14 ± 0.05 to 1.51 ± 0.09 and 2.33 ± 0.15 respectively. The lactate increase being larger in DM-A than in DM-S rats.

In conclusion the effect of DM on the changes in pancreatic hormonal secretion and muscular glycogenolysis during exercise is not secondary to changes in overall metabolism. During exercise adrenaline acts as an acute enhancing effect on muscular glycogenolysis, glucagon secretion and an acute depressing effect on the lactate secretion.

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**Estimation of oxygen uptake, heart rate and rectal temperature in running rats**

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It is extremely useful as an experimental tool in exercise physiology. Nevertheless there is shortage of information about the relationship between work intensity and basal physiological parameters obtained in steady state in exercising rats.

We have now developed a metabolic chamber including a treadmill for determination of oxygen uptake ( $\dot{V}_{O_2}$ ), heart rate (HR), rectal temperature ( $T_r$ ) and rectal temperature ( $T_r$ ) in running rats. Furthermore, arterial blood sampling and intravenous infusion can be made through specially implanted catheters.

In running rats  $\dot{V}_{O_2}$  and HR were 2.3 ± 0.2 ml  $O_2$  (ml  $100\text{ g}^{-1}$  min $^{-1}$ ) and 394 ± 10 beats  $\text{min}^{-1}$  respectively.

Determined rate was at an inclination of 0° and running speed of 10 m  $\text{min}^{-1}$  for 31 min. During the initial 15 min  $\dot{V}_{O_2}$  and HR decreased from 4.3 ± 0.3 ml  $O_2$  (STPD) (ml  $100\text{ g}^{-1}$  min $^{-1}$ ) and 412 ± 21 beats  $\text{min}^{-1}$  and 8.9 ± 0.03 in the 7th min to 4.3 ± 0.2 ml  $O_2$  (STPD) (ml  $100\text{ g}^{-1}$  min $^{-1}$ ) and 421 ± 17 beats  $\text{min}^{-1}$  in the 15th min (0.05) whereas  $T_r$  remained unchanged 36.8 ± 0.3 °C. This last "tail overheat" did not diminish during the four times the rats within a period of 10 days participated in exercise experiments. None of the measured parameters changed from the 15th to the 31th min. After 11 min of exercise the inclination was

on 0 5 10 or 15 and the speed was stepwise increased with 5 m  $\text{min}^{-1}$  every 11 min. At all inclinations  $\dot{V}_{O_2}$ , HR and  $T_r$  increased significantly with increasing running speed. No levelling off of  $\dot{V}_{O_2}$  and HR was observed. The highest measured  $\dot{V}_{O_2}$ , HR and  $T_r$  were 8.6 ± 0.3 ml  $O_2$  (STPD) (ml  $100\text{ g}^{-1}$  min $^{-1}$ ), 336 ± 16 beats  $\text{min}^{-1}$ , 0.92 ± 0.02 and 40.0 ± 0.4 °C respectively. No differences in  $\dot{V}_{O_2}$ , HR, and  $T_r$  were found between experiments at different inclinations and identical speeds.

The present study has shown the in order to carry out work physiological investigations in rats it is necessary to introduce a 20 min pre-test period during which unspecific stress responses to handling and new environment disappear. This pre-test period can not be replaced by "habituation runs on 3 preceding days". Furthermore, using rate running speed rather than inclination has to be changed in order to establish physiologically significant differences in work intensity. Taking these findings into account the developed metabolic chamber makes it possible to carry out sophisticated studies of metabolic, hormonal and cardiovascular adaptations to exercise in rats and to relate the responses to oxygen uptake (Bomze et al.).

#### Ref. 1979

JOSE & RICHTER E.A. GALBO R. Acta Physiol Scand Suppl. 341 volume

#### D 50

##### Function and reactivation of adenosine in fat cells. Effect of propylthiouracil (PTU) treatment.

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In cells from hypothyroid animals are less responsive to lipolytic stimuli than cells from euthyroid animals. The mechanism behind this defect is not studied. It does not involve decrease in the number of  $\beta$ -adrenoceptors or adenylyl cyclase, nor an increase in phosphodiesterase, but may involve defect in the coupling between the  $\beta$ -receptor and the cyclase (Malbon et al. 1978). Recently, Chisalo & Stouffer (1978) showed that adenosine deaminase (ADA) which inactivates adenosine that does cause a coupling between the receptor and the cyclase (see Fredholm 1978) restored the lipolytic sensitivity to adrenaline. The adenosine antagonist theophylline has a similar effect (Malbon et al. 1978). Thus, isolated rat fat cells we have therefore examined the possibility that drug-induced hypothyroidism (PTU in the drinking water for three weeks) is associated with change in the formation or metabolism of adenosine.

PTU-treatment shifted the dose-response curve to noradrenaline (NA) about one order of magnitude to the right. In agreement with the above mentioned results ADA (1 U/ml) did restore the lipolytic sensitivity to normal in the fat cells from treated rats. However, in the control fat cells ADA caused a shift of the dose effect curve to the left so that the difference between control and PTU-cells remained even in the presence of ADA.

The rate of accumulation of adenosine+inosine was

higher in the cells from PTU-treated rats than in control cells under basal conditions (115±20 vs 44±8 pmol/30 min/10<sup>6</sup> cells). In the presence of NA (1  $\mu$ M) the accumulation was higher in both groups (140±15 vs 82±15).

Adenosine is metabolized either by deamination to inosine and further or by uptake into cells and phosphorylation to AMP, ADP and ATP. The latter process had  $K_m$  of 1.2  $\mu$ M and  $V_{max}$  of 3-8 pmol/min/10<sup>6</sup> cells. There were no significant differences between control and PTU-cells. The deaminating activity of the bovine serum albumin used in the incubation was much higher than that of the fat cells. Under ordinary incubation conditions (100 000 cells/ml, 33 albumin) the activity contributed by the albumin was about 15 times higher than that present in the fat cells.

The present results indicate that drug-induced hypothyroidism is associated with an increased rate of adenosine formation, with little or no change in adenosine metabolism. An increased influence of adenosine may contribute to the well-known  $\beta$ -adrenoceptor subsensitivity in hypothyroidism, especially if the sensitivity of cells to adenosine is increased (Chisalo & Stouffer 1978).

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# Sympathetic regulation of adenosine in the rabbit heart

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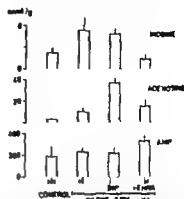
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Adenosine is considered a physiologically important coronary vasodilator (Berne & Rubio 1974). Adenosine may also act as an inhibitor of the inotropic and metabolic effects of catecholamines (Schradner et al 1977) and of the release of catecholamines from cardiac nerve endings (Hedqvist & Fredholm 1979). We report the frequency-dependent increase in adenosine levels and overflow by sympathetic nerve stimulation in rabbit hearts.

Rabbit hearts were perfused according to Langendorff with oxygenated Tyrodes solution and their sympathetic nerve supply were stimulated (Hedqvist & Fredholm 1979). The perfusates were continuously collected and analyzed for purine nucleoside content by reversed phase high performance liquid chromatography. In addition hearts were freeze clamped and contents of adenosine, inosine, guanosine and AMP assayed.

The effect of sympathetic nerve stimulation (10 Hz, 2 min) on cardiac levels of adenosine, inosine and AMP are shown in the Fig. A 3-fold increase in cardiac adenosine with no significant alteration in AMP is shown. In the presence of dipyrindamol a further 3 fold and in the presence of an adenosine deaminase inhibitor (EHNA) a 15-fold increase in adenosine content is seen. Overflow of adenosine and inosine into perfusates was increased by nerve stimulation. The increase was related to frequency

## PURINES IN RABBIT HEART



(1.25-10 Hz) and length of stimulation (0.5-2 min). Overflow of adenosine was markedly enhanced by dipyrindamol and EHNA. The amounts of adenosine appearing in the perfusates are similar to those required to produce significant inhibition of noradrenaline release and of cardiac catecholamine actions supporting a feedback regulatory role of adenosine.

These studies were supported by Swedish Medical Council Research (2553/4342) Karolinska Institutet & Magnus Bergvalls foundation.

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## D 52

Similarities between synaptic action of Ib tendon organ afferents and Ia muscle spindle afferents to spinal motoneurons

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Group I inhibition of homonymous and synergistic motoneurons and group I oligosynaptic excitatory have been attributed to the action of Ib tendon organ afferents. Recently it has been shown (Fitz et al 1979) that group I muscle spindle afferents can also contribute to the autogenic inhibition of homonymous motoneurons. The present results tend to substantiate and show that Ia afferents contribute both to the inhibition of synergist and to the oligosynaptic excitation of motoneurons as well.

Since small (<40 µm) brief triangular stretches of triceps selectively activate the Ia afferents (Brown et al 1967) this technique was used to evoke synaptic actions of Ia afferents in the motoneurons which innervate the following muscles of the cat hindlimb: post-biceps-semi-tendino-gracilis, semi-membranosus, biceps, quadriceps, tibial flexor digitorum longus, and peroneus tertius and brevis. Under chloral anaesthesia and muscle paralysis the triceps was tonically stretched to an initial tension of 5 N, the brief stretches applied and intracellular effects recorded from lumbar motoneurons. A signal averager aided in the detection of small amplitude postsynaptic effects.

The results demonstrated that a variety of oligosynaptic effects result from activation of only the Ia muscle spindle afferents. The segmental latencies of these effects (1.1-4 ms) are compatible with direct and trisynaptic linkages in these pathways (Eccles et al 1957). IPSPs were evoked in large proportion of the extensor motoneurons, while EPSPs predominated in the flexor motoneurons. This pattern is similar to the one described for the actions of Ib tendon organ afferents (Eccles et al 1957). A number of motoneurons were found which displayed both oligosynaptic IPSPs and EPSPs; a response not described for actions of the Ib afferents. The results together with previous observations on the convergence of group Ia and Ib afferents upon central interneurons (Jankowska et al 1978) suggest that common interneurons may be involved in mediating the action of Ia and Ib afferents to motoneurons.

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 during the permeability of the capillary  
 bed as an index of perfused micro-  
 vascular exchange area  
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As shown by Krogh (1919) the number of open  
 microcapillaries per unit cross section  
 are larger during exercise than during  
 rest. The degree of this recruitment  
 is controversial. In the present study  
 of recruitment was examined in the cat  
 using the isolated gastrocnemius prepara-  
 tion which provides ingoing and outgoing  
 arterioles and non-circulating  
 system.  $PS = CMC$  was determined by the  
 pulse injection method using sodium de-  
 duction (indicators Cr EDTA & Co-  
 all) and venous sampling (C indulin).  
 The response curves were analyzed as de-  
 scribed earlier (Sajersen 1970, 1979, Pas-  
 sen & Nielsen 1976). If it is assumed that  
 the average distance between perfused ca-  
 pillaries is 100  $\mu m$  then the average  
 intracapillary radius is 3.2  $\mu m$  and the  
 average capillary length is 1200  $\mu m$ . We  
 can estimate 1) capillary surface area  
 per g tissue  $S = 2\pi r L$  ( $cm^2/g$ ) 2) capillary  
 open pore area per g tissue  $S_{pore} = PS \Delta x / D$   
 ( $cm^2/g$ ) 3) number of perfused capillaries  
 per g tissue  $N$  4) intercapillary distance  
 ( $cm$ ) 5) volume of open capillaries per g  
 tissue  $V = \pi r^2 L$  ( $cm^3/g$ )  $\Delta x$  is the diffu-

fusion distance across the membrane (1000  $\text{\AA}$ )  
 and  $D$  is the free diffusion coefficient in  
 water. Experimentally it was found that  
 $PS/D = CMC/D = 53 \pm 3 \text{ (} \mu m^2 \text{ min)}^{-1}$  ( $n = 10$ )  $p < 0.01$  if  
 plasma flow is  $ml/100g \text{ min}$  see Fig 1  
 where the asymptote presented the in-  
 dication that in skeletal muscle recruit-  
 ment of capillaries is a linear function  
 of plasma flow. When plasma flow increases  
 from 1 to 30  $ml/100g \text{ min}$  recruitment takes  
 place with a factor of 4  $\pm 4$  times  
 more capillaries are perfused during heavy  
 exercise than during rest.

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#### D54

Prepared rectified smooth EPG of single skeletal  
 muscle unitary up to 100 Hz

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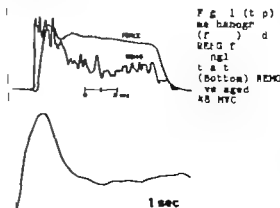
It has been demonstrated that the rechan-  
 ges of brack maximum 1 t y was 1  
 contraction (MVC) 1 b phase 1  
 wave ppe pp inset ly 337 ms  
 after the contraction initiated  
 (Secher et al 1978 Rube & Secher  
 1979). The present study describes the  
 rectified smooth EPG (REMG) of the  
 wave averaged real content of the  
 six healthy subjects and of four  
 with previous pe in the forearm  
 present performed pe in the thumb p  
 are MVC once every fifth second for  
 1.5 sec. Force was registered on a  
 force dynamometer and order on  
 with the neural frequency of 293 Hz REMG  
 was recorded (Fig 1 top) or  
 frequency modulated and recorded on a  
 recorder. The 48 REMG was a ragged  
 signal peaking at 1000 Hz compo-  
 sition pass filter on following the time  
 constant 107 msec.

The notch in the wave hange occurred  
 70 msec (Fig 1 top). The REMG showed  
 large amplitude at the beginning of the  
 contraction followed by a plateau  
 lower level. When averaged over the 48  
 REMG the only peak was the REMG  
 disappeared (Fig 1 bottom). For the 1

subject the peak REMG amplitude ppe d  
 t 157  $\pm 3$  (E) ms c t h ed t  
 310  $\pm 47$  ms and the plateau had  
 amplitude of 63  $\pm 8$  % of the peak  
 The ultimate value at that time was 1  
 br 1 i ed 1 tabl hing th fi t  
 part of the MVC was the large  
 EPG ppe the 1 fib re  
 position of the re t f th t ti

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# Sympathetic regulation of adenosine in the rabbit heart

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Adenosine is considered a physiologically important coronary vasodilator (Berne & Rubio 1974). Adenosine may also act as an inhibitor of the inotropic and metabolic effects of catecholamines (Schrader et al 1977) and of the release of catecholamines from cardiac nerve endings (Hedqvist & Fredholm 1979). We report the frequency-dependent increase in adenosine levels and overflow by sympathetic nerve stimulation in rabbit hearts.

Rabbit hearts were perfused according to Langendorff with oxygenated Tyrodes solution and their sympathetic nerve supply were stimulated (Hedqvist & Fredholm 1979). The perfusates were continuously collected and analyzed for purine nucleoside content by reversed phase high performance liquid chromatography. In addition hearts were freeze clamped and contents of adenosine, inosine, guanosine and AMP assayed.

The effect of sympathetic nerve stimulation (10 Hz, 2 min) on cardiac levels of adenosine, inosine and AMP are shown in the Fig. A 3-fold increase in cardiac adenosine with no significant alteration in AMP is shown. In the presence of dipyridamol a further 3 fold and in the presence of an adenosine deaminase inhibitor (EHRA) a 1.5-fold increase in adenosine content is seen. Overflow of adenosine and inosine into perfusates was increased by nerve stimulation. The increase was related to frequency

PURINES IN RABBIT HEART



(1-25-10 Hz) and length of stimulation (0.5-2 min). Overflow of adenosine was markedly enhanced by dipyridamol and EHRA. The amounts of adenosine appearing in the perfusates are similar to those required to produce significant inhibition of noradrenaline release and of cardiac catecholamine actions supporting a feedback regulatory role of adenosine.

These studies were supported by Swedish Medical Council Research (2553 4342) Karolinska Institutet & Magnus Bergvall's foundation.

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## D 52

Similarity between synaptic action of Ib tendon organ afferents and Ia muscle spindle afferent upon pin1 motoneuron

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Group I inhibition of homonymous and synergistic motoneurons and group I oligosynaptic excitation have been attributed to the action of Ib tendon organ afferents. Recently it has been shown (Fetz et al 1979) that group Ia muscle spindle afferents can also contribute to the autogenetic inhibition of homonymous motoneurons. The present results tend to corroborate and show that Ia afferents contribute both to the inhibition of synergists and to the oligosynaptic excitation of motoneurons as well.

Since small (< 40 µm) brief triangular stretches of triceps muscles selectively activate the Ia afferents (Brown et al 1967) this technique was used to evoke synaptic actions of Ia afferents in the motoneuron. A high innervated flexor muscle of the hindlimb, posterior biceps semitendinosus, gracilis, semimembranosus, biceps quadriceps, tibial flexor digitorum longus and peroneus tertius and brevis. Under chloral anaesthesia and muscle paralysis the triceps muscle was tonically stretched to an initial tension of 5 N, the brief stretches applied and intracellular effects recorded from lumbar motoneurons. A signal averager aided the detection of small amplitude postsynaptic effects.

The results demonstrate that variety of oligosynaptic effects result from activation of only the Ia muscle spindle afferents. The segmental latencies of these effects (1-4 ms) are compatible with direct and trisynaptic linkages in these pathways (Eccles et al 1957). IPSPs were evoked in a large proportion of the tested motoneurons. The EPSP predominated in the flexor motoneurons. This pattern is similar to the one described for the actions of Ib tendon organ afferents (Eccles et al 1957). A number of motoneurons were found which displayed both oligosynaptic IPSPs and EPSPs, a response not described for actions of the afferent. These results together with previous observations on the convergence of group Ia and Ib afferents upon certain interneurons (Jankowska et al 1978) suggest that common interneurons may be involved in mediating the actions of Ia and Ib afferents to motoneurons.

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lative gamma-radiation autoradiography for flow measurement

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Local blood flow can be measured using several different procedures e.g. dye records, lectro- and ultrasonic flow meter, plathysmography and during the past two decades by different methods of arterial diffusible and non-diffusible substances injected in the vascular system or the subject to study. Using e.g. the microsphere trace technique lumps of tissues of interest are dissected out and analysed for radioactivity. These lumps are rarely subject to histological study and often histogeneticities regarding morphology and function are overlooked. This is mainly true in the study of tumour circulations where such heterogeneities generally are of theoretical and clinical importance. The microsphere trace technique was thus further developed to allow: 1. recording of partial heterogeneities 2. correlation of perfusion with histology 3. quantitation of blood flow in selected discrete regions of tissue. After injection of labelled spheres and drawing reference blood tissues e.g. later are dissected out and fixed in formalin. Two mm thick slices are cut and placed upon an X-ray film. One plain or combined with intensifying screens. The reference blood is hypercalorized and finally diluted. In each dilution the spheres are allowed to settle onto standardized areas of

glass film. The film is placed together with the tissue sections on the X-ray film. After an exposure time of weeks the densitometric blackening from the tissue section can be compared with the reference blackening and blood flow at any point can be expressed as volume blood/weight of fixed tissue and the histological sections can be produced from the fixed autoradiographed section provide morphological correlation.

Two different isotopes can be used and analysis is accomplished either by radiation quality discrimination (e.g.  $^{125}\text{I}$  &  $^{131}\text{I}$ ) or by temporal discrimination (e.g.  $^{125}\text{I}$  &  $^{99\text{m}}\text{Tc}$ ).

The procedures thus allow identification of heterogeneous perfusion, quantitation of blood flow in any desired region as well as morphological correlation to functional data.

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#### D 58

soler reactivity experimental mammary tumours and normal vascular beds studied in vivo with microsphere perfusion

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In vivo studies with microsphere techniques (Weiss et al. 1979) on induced and autotransplanted DMBA (dimethylbenzanthracene) rat mammary neoplasms have demonstrated that the tumours have a relatively high resting blood flow and strong response to noradrenaline indicating relatively low resting tone in the tumour vascular bed. Skin to which the tumours are histogenetically related show under the same conditions low resting flow and slight response to noradrenaline indicating high resting tone. Due to the varying reactivity resting vascular tone, the different organs in the in vivo situation it is difficult to evaluate the relative reactivity to pressor drugs against this background. It is advantageous to use the in vitro artificial perfusion technique where the haemodynamic characteristics can be subject to more detailed analysis from maximal vascular relaxation to maximal constriction. Thus excised rats with DMBA induced mammary neoplasms were artificially perfused and microspheres with three different isotopes were injected at different points of the pressure/flow and dose/response curves for infused noradrenaline starting at maximal relaxation. By using the microspheres the flow distribution relationships between the different organs could be studied. It was shown that the tumour vascular bed had pressure/flow

curve during maximal relaxation which was located between that of skin and that of muscle. During temporary noradrenaline infusion the tumour vascular bed responded more than any of the other vascular beds studied such as muscle, skin, liver, salivary gland, kidney, spleen and uterus. The organ which was lowest in characteristics was skin.

It was concluded that the blood vessels supplying the DMBA induced mammary tumours have reactivity during maximal relaxation with the range of such vascular beds as muscle and skin and that they are extremely reactive to noradrenaline even as compared to their histogenetical origin skin. Whether this increased reactivity to noradrenaline is due to the lack of nerve endings remains to be investigated.

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The effect of salmon calcitonin (SCT) on the total plasma calcium in a marine teleost fish

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Calcitonin (CT) is known to be produced in the ultimobranchial gland and present in relatively high concentrations in the blood of both bony fish and elasmobranchs.

Although fish calcitonins show very high potency in producing hypocalcemia in mammalian experiments on fish have mostly given negative findings. Thus salmon calcitonin (SCT) does not affect the calcium of salmonids (Pang 1971, Copp et al 1972, Milhaud et al 1977) and neither does salmon calcitonin affect the calcium of the Japanese eel (Yamuchi et al 1978).

A series of experiments have been carried out to test the effect of SCT (SANDUZ) on the calcium in the Atlantic cod *Gadus morhua* L. Doses of 5.0, 10<sup>-9</sup>, 1.0, 10<sup>-8</sup> mol/kg were used and injected i.m. or i.v. at t 0. Group size varied from 5-8 and control groups received corresponding amount of the SCT solvent.

Exp. I and II were carried out on fish in normal sea-water ([Ca] 10 mM). Exp. I tested for any short-term effect on calcium (blood samples taken at t -60, 0, 10, 30, 60, 120, 240 min) and exp. II tested for any long-term effect (blood samples taken at t 0, 6, 24 hrs).

Exp. III and IV were carried out on fish placed in hypercalcemic sea-water ([Ca] 100 and 150 mM respectively) at t 0 and blood samples taken at 2 hr intervals from t 0-10 hrs and at t 24 hrs.

Exp. IIb was identical to exp. III, except that the SCT dose was given at t 6 hrs instead of t 0.

No change in calcium was noted in exp. I and II. In exp. III and IIb both the experimental and control groups developed hypercalcemia (from 2.5 mM up to 3-4 mM) within 2 hrs, which persisted throughout the exp. No effects of SCT were noted either in exp. III or exp. IV. The calcium increased steadily in the control group till death occurred whereas in the experimental group the increase halted at t 6 hrs and no further increase occurred between t 6-10 hrs. All fish in both groups died within t 24 hrs.

The following can be concluded: a) CT does not play a part in preventing hypercalcemia and its endogenous production may become a limiting factor under extreme conditions. b) The endogenous content of the calcitonin is very strict as CT is unstable under stable conditions (normo- or hypercalcemic).

Experiments designed to study the effect of a long-term SCT treatment of the cod and the reactions of these fish to hypercalcemic environment are now in progress in this laboratory.

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#### D 56

Inhibition of epoxide hydrolase by heavy- and organometals in vitro

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The effects of Cd, Hg<sup>++</sup>, Cu<sup>++</sup>, Zn, Ni<sup>++</sup>, Co<sup>++</sup>, Cr<sup>++</sup> and Ag, as well as methylmercury, dimethylcadmium and p-chloromercuribenzoate were studied on rat hepatic microsomal epoxide hydrolase (EH) (styrene oxide, SO, benzo(a)pyrene-4,5-oxide, BPO) in vitro.

1. The effect of buffer and pH used was critical. Cadmium was strongly inhibitory only in tris buffer pH 9 but not in phosphate buffer pH 7 or carbonate buffer pH 9. Inhibition by Zn and Ni was seen in carbonate and tris buffers pH 9 but not in phosphate buffer pH 7. The inhibition by Hg and Cd was stronger in phosphate and carbonate buffers than in tris. Cd and Cr were poor inhibitors in the conditions tested. Ag was strong inhibitor of EH (as well as monooxygenases) but poor inhibitor of glutathione S-transferase.

2. Some of the metals were scavenged strongly from

the medium by microsomal membranes. This was concluded from the facts that: a) Dilution of the enzyme preparation (microsomes) increased the inhibitory potency. b) Hydration of BPO (which as a sensitive method needs only one 40th part of the enzyme amount needed in measurement of SO hydration) was much more sensitive to these metals than hydration of SO (a common enzyme is involved). Especially sensitive to the amount of microsomal lipid present in the incubation were Ag, Hg and Cd. 3. Methylmercury and dimethylcadmium were also inhibitory to EH. The inhibition was much more pronounced in tris (pH 9) than in phosphate buffer (pH 7). p-Chloromercuribenzoate inhibited the hydration of BPO potentially in phosphate buffer (pH 7) but only slightly in tris buffer (pH 9). Strong binding to microsomal lipid was likewise evident.

The present data indicate that many heavy metals and organometals are inhibitors of epoxide hydrolase. Detection of the inhibitory effect is strictly bound to the conditions used.

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primary projections from different types of principal cells to perigeniculate neurons.

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Principal cells in the lateral geniculate nucleus receive inhibition through subcortical recurrent pathway via synapses upon collaterals of principal cells and inhibitory interneurons in the perigeniculate nucleus. A convergence of excitation from different types of principal cells onto perigeniculate neurons has now been investigated in acute experiments on primate geniculate.

Two types of perigeniculate cells were found. One type was excited from sustained, slowly conducting principal cells; the other from transient, rapidly conducting cell types received convergent connections from "on-centre" and "off-centre" principal cells. Their receptive fields were round and about twice the size of the receptive field for the corresponding type of principal cells.

In contrast to the principal cells the majority of the perigeniculate neurons were binocular indicating that they receive excitation from principal cells in both eyes. A<sub>1</sub> and A<sub>2</sub> of the lateral geniculate nucleus. The perigeniculate cells in turn had axonal branches which innervate both eyes.

These findings demonstrate that visual signals from both eyes and from both the "on-centre" and the "off-centre" systems are mixed at the interneuronal level in a recurrent inhibitory pathway to principal cells.

## D 62

Alcain sensitivity of isolated mesenteric resistance vessels in spontaneously hypertensive rats

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Studies on isolated arteries (Barnes & Mulvany 1976) and resistance vessels (Mulvany et al. 1979) show that if neuronal uptake is inhibited the noradrenaline (NA) sensitivity of SHR vessels is greater than that of WKY vessels. In the present study we have investigated possible causes for the increased NA-sensitivity.

Small mesenteric arteries (internal diameter ~150 µm) from 3-4 month old SHRs and WKY rats mounted as described previously (Mulvany & Halpern 1976) but here on a small myograph permitting paired experiments. Vessels were depleted of Ca<sup>++</sup> by repeated titration in Ca-free solution (containing 0.1 mM EGTA). They were then exposed successively to solutions containing 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 2.5 µM Ca<sup>++</sup>. All solutions also contained 3 µM cocaine to prevent neuronal uptake. They were held in each solution for a period of 4 min being maximally activated during the second half of each period by the addition of 10 µM NA.

In each of 5 paired experiments the SHR vessel responded to lower Ca-concentrations (Ca ED<sub>50</sub> 0.10 ± 0.02 mM (SE)) than

the WKY vessel (Ca ED<sub>50</sub> 0.18 ± 0.03 mM). Corresponding experiments with K-stimulation (with 1 µM phenolamine for α-receptor blockade) showed by contrast little difference between SHR and WKY vessels either in their Ca-sensitivity or in their Ca-sensitivity in respect to K-stimulation.

The results suggest that the greater NA sensitivity of the SHR vessels compared with the WKY vessel is due not so much to differences in the calcium permeability of their plasma membranes but rather to a specific difference in the effectiveness of their adrenergic receptors in utilizing extra- and intracellular calcium.

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Emotional stress increase plasma adrenaline and cyclic AMP in unrestrained rats  
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Plasma cyclic AMP and catecholamines were determined on samples withdrawn from unrestrained rats via chronically implanted aortic catheters. The rats were trained to acquire a conditioned avoidance reflex in two-way shuttle box. The unconditioned stimulus was an electric shock (0.5-1 mA) delivered every 1 x 5 ml via metal rods forming the floor of the box. The conditioned stimulus consisted of sound and light signal delivered 5 s before the electric shock.

Initial experiment (rats conditioned with 1 mA shocks every 5 min) showed significant increases in plasma cyclic AMP from 12.1 ± 2.2 to 16.3 ± 4.4 (n=7, p<0.05) after 30 min stress with no change in controls. In second set of experiment (rats conditioned with 0.5 mA every min) plasma cyclic AMP increased less. Fig. 1 shows the relationship between arterial plasma catecholamines and cyclic AMP in these animals. It can be seen that low resting levels of catecholamines prevailed and that the increased adrenaline but not noradrenaline levels. There was correlation between level of cyclic AMP and adrenaline but not noradrenaline in plasma. Our results suggest that adrenaline may be of major importance for the increase in plasma cyclic AMP seen in rat during stress. This is in agreement with observations that adrenaline is considerably more potent stimulator of plasma cyclic AMP than

1 noradrenaline. Experiments with  $\beta$ -blocking drugs progress to further define the role of adrenaline. The cyclic AMP response to stress. Work is also under way to study the effects of different kind and intensities of stress on the secretory response from sympathetic nerves and adrenal medulla.

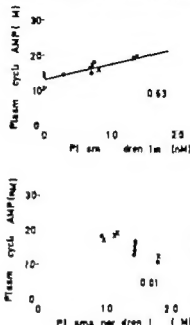


Fig. 1: Relationship between arterial plasma catecholamines and cyclic AMP in rats during (x) and before or after (o) conditioned avoidance stress.

## D 60

A Comparison of Skeletal Muscle Morphology, Metabolism and Function in Smokers and Non smokers  
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The impaired physical performance capacity in tobacco smokers is probably partly related to the effects of smoking on the muscle tissue itself. This seems conceivable since physical working capacity and metabolic capacity of the working muscles are closely interrelated (Holloszy & Booth 1976). It was therefore thought to be of interest to compare a group of smokers and non-smokers from a homogenous population of men with respect to skeletal muscle morphology, metabolism and function. This population had earlier been used to study the effects of age on these variables (Larsson 1978).

Forty-three healthy males (18 smokers and 25 non smokers) all white-collar workers volunteered for the study. Samples were taken from the vastus lateralis muscle for histochemistry, enzyme assays and electron microscopy using the needle biopsy technique. The muscle fibres were stained for myofibrillar ATPase and NADH activity. The muscle fibres could then be classified into type I, IIA, IIB and IIC fibres and their fibre area determined. Activities of muscle enzymes representing key enzymes of skeletal muscle energy metabolism were measured (PFK, LDH, CS, CytOx and NAD). Stereological analyses of the mitochondria were performed on electron microscope micrographs. In the knee extensor muscles the maximum isometric and dynamic

strengths were measured at velocities corresponding to 0°, 60°, 120° and 180° x s<sup>-1</sup> using an isokinetic dynamometer.

The proportion of type I fibres was lower (p<0.001) in smokers compared to non smokers (36% vs 50.7 ± 2.5%). Type II fibre subclassification revealed a correspondingly increased incidence (p<0.01) of type IIB fibres in the smokers. Fibre areas did not differ in the two groups. Muscle oxidative capacity was lowered in the smokers as judged from decreased (p<0.05-0.001) mitochondrial enzyme activities and a lowered (p<0.01) fibrillar space mitochondrial volume fraction. Isometric and dynamic strengths were lower (p<0.05-0.01) in the smokers except at the highest velocity of movement studied.

The present results show some clear-cut differences in skeletal muscle morphology, metabolism and function between smokers and non smokers. The reduction in muscle oxidative capacity and strength in the smokers may at least partly be explained by the difference in fibre type distribution. These differences may either be explained as an effect of smoking per se (e.g. the stimulating effect of nicotine on the muscle fibres at the motor end plate and its interference with acetylcholine metabolism (Fische et al 1960)) or it may be that background factors are responsible such as genetic differences.

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of the vasoactive intestinal polypeptide  
[a] by intracocular pressure (IOP) and regional  
blood flow

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Stimulation of the facial nerve causes  
afflux in the choroid and rise in IOP.  
This effect seems to be mediated by non-cholin-  
ergic fibers (Rasmussen & Ell unpublished  
data). Histochemical studies by Uddman et al  
(unpublished communication) have indicated that  
there are VIP-containing neurons in the facial  
nerve. VIP was discovered and named on the basis  
of its vascular effects (Said & Mutt 1970).  
The physiological role is not known (Said 1975).  
We have investigated the effects of intra-  
ocular as well as intravenous injections of VIP on  
IOP and the regional blood flow measured  
with labelled microspheres. Intravenous as well  
as intracocular injections in rabbits caused  
a rise in IOP at doses not affecting the blood pressure.  
At a dose of 500 ng/kg b.w. given over-  
intravenously there was little change in  
arterial blood pressure but marked vasodila-  
tion in several tissues. Flow increments in the  
adipose gland and pancreas were more than 100 %  
100 % in heart muscle and 25-50 % in the  
kidney, choroid, placenta, thyroid and submandi-  
bular gland. No consistent vasodilation was ob-  
served in this domain in the rat.

liver, adrenals, skeletal muscle and skin. Injec-  
tion of VIP into the anterior chamber caused marked  
vasodilation in the anterior uvea.

The results indicate that VIP at low doses pro-  
duces selective changes in the distribution of the  
cardiac output, which may be of therapeutic inter-  
est. The effects in the eye suggest that if VIP is  
liberated in the eye during facial nerve stimulation  
it may well be responsible for the vasodilation  
caused by the stimulation.

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# Ehaustion in reindeer calves

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Reindeer management at times especially in the coral implies a high train on the animals and certain loss of animals may occur. Optimal weather conditions may reduce this risk. It has been speculated on why reindeer eat an animal with highly developed running skills sometimes lay down for one or several reasons dying to stand on its feet. Some of the animals will finally die. Hyperthermia collapse has been used as an explanation for at least some of these cases of death.

Having had access to hardworking reindeer during tagging of calves in July, rectal temperatures of fasted calves were measured 7-10 cm into the rectum by means of a thermistor which was calibrated against a precision thermometer. The animals were running in a corral during tagging. Only the calves may obtain some food in the pen. In this study I have concentrated on the calves, the high risk group concerning collapse from exhaustion. Rectal temperatures of 20 males and 30 females normal calves registered at intervals of a five hour tagging period were:  $40.7 \pm 0.7$  (SD) °C and  $40.8 \pm 0.5$  (SD) °C respectively. A trend of decrease in temperature with time in the pen was seen in the data. Irrespective of the mean

temperature of the first 15 registrations  $41.1 \pm 0.4$  (SD) °C was compared with the mean of the 15 last registrations  $40.2 \pm 0.8$  (SD) °C. The decrease in mean temperature of 0.9 °C was significant:  $p < 0.001$  as tested by Student's t test.

Finally rectal temperature of 13 collapsed calves was  $39.3 \pm 0.4$  (SD) °C, highly significantly below the mean value of all the more normal groups referred to above:  $p < 0.001$  when compared with the 15 last registered (Student's t test). These 13 calves showed the typical symptoms of exhaustion and data from these are included in the more normal group referred to above.

In one of the calves which died from exhaustion dissection for fat stores revealed a complete lack of fat in the axillar, inguinal and renal regions, areas where some fat is usually found.

These data indicate that hyperthermia is not the main cause of mortality in these exhausted animals as they have lower core temperature than controls. Exhaustion however is not yet a well defined condition in any mammal. The problems of limiting factors in a heat stress are far from solved. Depleted energy reserves obviously is a factor of importance. When looking for the causes of death in exhausted reindeer congestive heart lung edema and shock should of course be taken into consideration. In addition depletion of available nerve

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The blocking effect of diphenylhydantoin on parasympathetic nerve outflow treated rat phrenic nerve diaphragm preparation

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Pretreatment with 0.1 mM para-hydroxymercuribenzoate (pOHMB) increased the blocking effect of diphenylhydantoin (DPH) (0.5-1.0 mM) on the indirectly stimulated (0.1 Hz) rat diaphragm. The present experiments were performed to describe and localize this effect.

pOHMB is a SH-group inhibitor and the increased block was dependent upon the reaction between pOHMB and SH groups, since SH-group protection with dithiothreitol (Cleland 1964) abolished the combined effect of pOHMB and DPH.

The muscle contracted upon direct stimulation after the contraction to indirect stimulation had ceased. Tests on the isolated phrenic nerve did not reveal increased blocking effect of the combination of pOHMB and DPH. This indicated that the block should be localized to the neuromuscular transmission process.

A tenfold increase in the  $Ca^{++}$  concentration slightly increased the block. This indicated that the block was not due to competition with the  $Ca^{++}$  flux into the nerve terminal leading to decrease of acetylcholine release. Decreased presynaptic excitability or decreased postsynaptic

receptor sensitivity may explain the  $Ca^{++}$  potentiation of the block.

In order to separate between a pre- or postsynaptic mechanism of action microelectrode technique with recording of miniature endplate potentials (MEPPs) and endplate potentials (EPPs) was used. MEPPs could be recorded after contraction had ceased, indicating that the block was due neither to acetylcholine depletion nor to inhibition of the receptor sensitivity. EPPs were recorded in the curarized preparation where the action potentials were abolished. The EPPs disappeared abruptly after a period of slight decrease of amplitude after DPH addition.

A significant time difference to cessation of endplate potentials between the pOHMB treated preparation and the preparation where DPH was added alone suggested that an interference with the excitability of the nerve terminal was the cause of the block.

### References

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